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AMERICAN DAIRY SCIENCE ASSOCIATION

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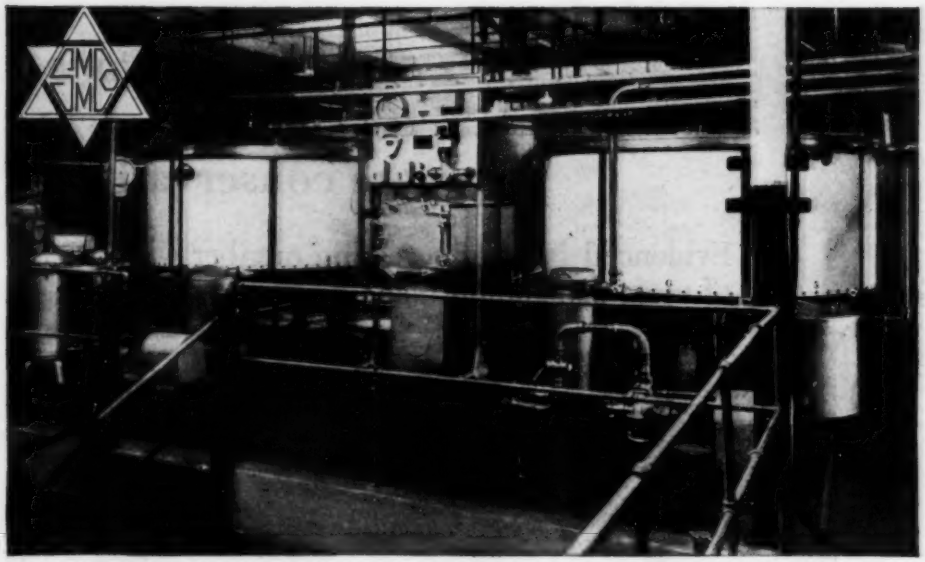

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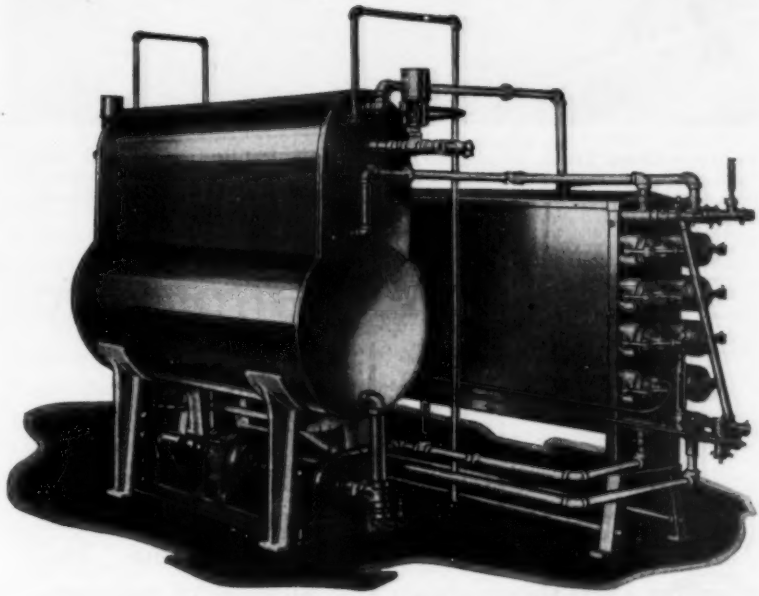

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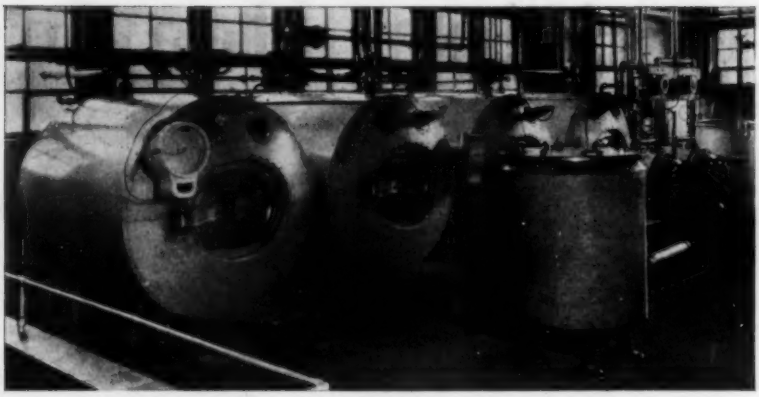

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NORMAL VARIATIONS IN THE CALCIUM CONTENT OF THE BLOOD OF DAIRY CATTLE*

L. S. PALMER AND C. H. ECKLES

Divisions of Agricultural Biochemistry and Dairy Husbandry, University of Minnesota, University Farm, St. Paul

In a previous paper (1) we have shown the extent to which the inorganic phosphate of the blood plasma of dairy cattle may vary from day to day. A biometric study of 60 sets of 3 consecutive-day analyses, taken at monthly intervals, revealed the fact that 76 per cent of the samples varied significantly from the theoretical values indicated by the coefficients of correlation of the respective days. Although the mean deviation of all actual values from the theoretical was only 14.3 per cent, several deviations exceeded 50 per cent and one reached 123 per cent of the expected.

Although the blood samples which furnished these data were taken primarily for the purpose of studying the phosphate concentration in the blood, the calcium content of the plasma was also determined. The data thus secured furnish the basis for the present report.

The literature to-date discloses the calcium content of about 350 samples of blood plasma or serum from presumably normal cattle (2–13) and over 100 samples from cattle suffering from milk fever (11–15). The great majority of the normal samples show a range of 9.0 to 12.0 mgm. per cent calcium with extreme values of 4.0 (8)[1] and 24.4 (5)[2] mgm. per cent, respectively. The lowest value so far reported (13) is 2.7 mgm. per cent for a cow "down" with milk fever. A great variety of dietary, physiological, environmental, and other conditions are represented among the animals that furnished the presumably normal samples.

* Received for publication February 7, 1930. Published with the approval of the Director, as Paper No. 925, Journal Series, Minnesota Agricultural Experiment Station.

[1] From a calf on a diet of whole milk and paraffin oil.

[2] From a lactating cow on a ration of timothy hay, corn silage, and grain mixture. The figure is comparable to the calcium content of whole blood in dairy cattle, but not to that in serum or plasma.

It is difficult to determine from this mass of data what normal variations may be expected to occur when the dietary and physiological conditions are presumably constant for an individual animal. It has been the practice, for the most part, to interpret the effects of dietary and other changes on the basis either of a single sample or the average of several single samples taken at arbitrary intervals. Robinson and Huffman (7) have attempted to determine the daily variation in the calcium of the blood of 4 cows on a uniform diet in a series of successive daily samples ranging from 5 to 8 days. They encountered one twenty-four hour fluctuation of 2 mgm. per cent in one eight-day series whose mean calcium was about 8.5 mgm. per 100 cc. of plasma. Their data, however, are too meagre for biometric analysis, although they are the only data so far published on blood calcium of cattle which deal with the variations under uniform physiological conditions.

Meigs, Blatherwick, and Cary (3) concluded from a study of 41 calcium determinations made by them that, "the concentration of calcium in the plasma of cows is quite constant. Small variations can be induced by varying the amount supplied with the rations, but the chief controlling factor is probably the concentration of bicarbonate in the plasma.[3] It is probable that the concentration of calcium tends to vary inversely with that of the bicarbonate." This explanation of the variations in plasma calcium was accepted by Hart and associates (5), but there seems to be little support for it in the data secured by Robinson and Huffman. The largest day-to-day fluctuations in calcium encountered in the short series of the latter investigators were not accompanied by any appreciable change in carbon dioxide. Conversely they record great changes in carbon dioxide without any corresponding change in calcium. Likewise there is no indication that calcium and carbon dioxide fluctuate inversely.

Salvesen and Linder (16) have demonstrated that changes in serum protein in human nephritis are paralleled by changes in

[3] The probable relation of calcium to bicarbonate concentration in blood plasma appears to have been pointed out first by Rona and Takahashi in 1913 (Biochem. Z., xlix, 371–380).

serum calcium, but that in experimental tetany in dogs following parathyroidectomy, the drop in serum calcium is not accompanied by any change in serum protein (17). Hastings, Murray, and Sendroy (18) believe that the formula

$$[CA] = 0.014\,[P] - 1.4$$

expresses the relationship between calcium and protein in blood serums, the calcium being in mM. per kilogram H_2O and protein in grams per kilogram H_2O. Peters and Eiserson (19) have confirmed the direct relationship between serum protein and serum calcium in human nephritis. On the basis of their results and the generally accepted idea that the concentration of calcium in serum is likewise dependent (inversely) upon the level of inorganic phosphate, Peters and Eiserson propose the formula

$$Ca = -0.225\,P + 0.556\ \text{protein} + 7^4$$

to express the relation between serum calcium and what they regard as the two principal controlling variants in the blood. However, the errors which may be involved in applying this formula may exceed 2 mgm. Whether similar approximate relations hold true for cattle blood remains to be determined.

EXPERIMENTAL

The data which we present at this time are a biometric study of 60 sets of blood samples, each representing 3 consecutive days, taken at monthly intervals from August to December, 1926. The animals used were all in the University dairy herd. Twenty-four different animals, in all, furnished the data. Five animals had only 1 period of 3 consecutive days, 10 animals had 2 periods, 1 animal had 3 periods, and 8 animals had 4 periods. The ages of the animals ranged from calves to mature cows, some of the latter being in milk. The diets used represented various planes of calcium and phosphorus intake.

The blood was drawn from the jugular vein at the same hour each day. It was allowed to flow into 100-cc. glass tubes con-

[4] In this formula Ca and P are milligrams per cent and protein is per cent concentration.

TABLE 1

Day-to-day variations in calcium in successive three-day periods per 100 cc. plasma

ANIMAL	PERIOD 1						PERIOD 2						PERIOD 3						PERIOD 4					
	Ca_1		Ca_2		Ca_3		Ca_1		Ca_2		Ca_3		Ca_1		Ca_2		Ca_3		Ca_1		Ca_2		Ca_3	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
E 33	13.17		11.30	11.57	12.01	12.56	15.77	16.15	13.11	13.30	11.21	11.21	12.35	12.35	12.35	12.35	12.73	12.92	12.64	13.02	13.11	13.20	12.35	12.54
E 73	13.17	12.28	12.46	13.70	11.57	11.39	14.63	14.63	12.44	12.64	13.49	13.11	13.30	13.30	12.92	13.30	11.97	12.16	11.97	12.16	12.35	12.35	12.35	12.35
E 74	10.50	10.50	10.86	10.86	10.14	11.03	11.78	11.78	11.59	11.59	12.16	11.78	11.78	11.97	10.92	11.01	11.97	12.16	10.45	10.54	11.40	11.01	9.88	9.88
E 75	12.46	12.46	11.92	11.92	11.75	12.64	14.92	14.92	14.25	14.44	14.63	14.63	12.92	13.11	12.73	12.92	13.87	14.25	13.20	13.11	12.73	13.11	11.78	11.78
E 91	11.83	11.66	11.57	11.75	11.92	12.28	12.92	13.11	12.35	12.73	12.92	12.92	10.64	10.83	11.40	11.59	13.11	13.11	11.21	11.01	11.97	11.97	11.59	11.78
E 92	14.10	13.72	11.28	11.28	12.78	12.78	12.54	12.54	13.30	12.73	12.73	13.30	11.21	11.40	12.92	13.11	11.97	11.97	11.11	11.11	10.45	10.45	9.88	10.07
E 93	13.16	13.34	12.03	12.22	11.09	10.53	14.63	14.44	13.30	13.68	15.58	16.15	17.10	17.10	16.15	16.15	16.15	16.15	13.30		12.92	13.11	13.20	13.20
E 94	12.78	13.34	13.16	13.16	12.60	12.97	15.58	15.77	13.68	13.30	16.72	16.15	17.10		14.92	15.20	15.96	15.96	11.40	11.59	11.40	11.49	9.97	9.97
E 84	15.48	14.95	14.24	15.12	14.59	14.77	12.35	12.35	11.97	11.97	13.30	13.39	11.11	11.40	9.88	9.97	9.97	9.97						
E 58	11.39	11.30	11.12		11.30	11.48	14.06	13.87	12.92	12.92	12.54	12.35												
E 68	15.04	15.04	13.63	13.16	11.66	11.84	11.59	11.59	10.92	10.92	10.83	10.83												
E 77	13.72	13.91	14.10	13.72	13.16	13.16	11.21	11.40	11.97	11.68	10.54	10.83												
E 80	15.84	16.55	16.37	16.55	14.68	14.50	14.25	14.25	13.68	13.11	13.68	13.68												
E 81	16.37	17.08	16.55	16.20	15.66	14.95	13.68	13.68	11.01	11.21	14.63	14.92												
E 83	13.35	13.53	12.46	11.92	12.46	12.10	11.97	12.16	11.49	11.49	11.78	11.97												
E 85	17.26	17.46	16.02	15.66	13.35	13.17	14.25	14.25	12.73	12.92	12.92	12.82												
E 86	15.04	15.60	12.78	13.16	12.22	12.22	11.01	11.01	10.45	10.45	10.07	10.16												
E 88	14.59	14.95	13.35	13.53	17.08	17.62	11.97	12.35	11.01	11.01	14.92	14.63												
E 89	11.28	11.28	11.84	12.22	13.16	13.34	12.16		10.26	10.26	10.26	10.45												
E 78	16.62	16.62	11.40	11.40	13.11	13.11																		
E 79	14.44	14.63	14.25	13.87	12.73	13.30																		
E 87	13.91	13.91	11.28	11.84	12.60	12.78																		
E 95	11.97	11.97	12.54	12.54	14.92	15.39																		
E 96	12.16	12.54	11.78	11.97	12.54	12.54																		

taining 1 cc. of a saturated solution of sodium citrate. A 50-cc. sample was drawn from each animal at each bleeding. The blood in each tube was mixed thoroughly with the sodium citrate solution immediately to prevent coagulation. It was then centrifuged to separate the corpuscles from the plasma. Sufficient plasma was pipetted off at once and preserved in a refrigerator until analyzed. In nearly all cases the analysis was begun the same day the sample was drawn.

The method of analysis employed throughout was the Clark and Collip (20) modification of the Kramer-Tisdall procedure. Duplicate analyses were made of all samples.

The complete data, including the duplicate analyses are given in table 1. In this table Ca is the milligrams of calcium per 100 cc. of plasma, the subscripts 1, 2, and 3 referring to the first, second, and third days, and the subscripts a and b to the duplicate analyses.

The coefficients of correlation of the respective days, calculated by the formula of Harris (21) are as follows:

$$r_{Ca_1Ca_2} = +0.762340 \pm 0.036471$$
$$r_{Ca_1Ca_3} = +0.624557 \pm 0.053111$$
$$r_{Ca_2Ca_3} = +0.627991 \pm 0.052737$$

It is apparent that the coefficients of correlation of the values obtained on successive days are fairly high when the data are considered as a whole. The coefficients, however, are considerably lower than for the inorganic phosphate of the same samples, as shown in our previous paper (1), where the coefficients ranged between +0.93 and +0.97. Inspection of the data in the table shows that there were marked variations from day to day. Using the same method for showing the percentage deviation of the actual values from the theoretical as was employed for inorganic phosphate in the previous paper (1), the following equations were derived for the calculation of these deviations.

$$Ca_2 = +2.459 + 0.762\ Ca_1$$
$$Ca_3 = +4.432 + 0.625\ Ca_1$$
$$Ca_3 = +4.908 + 0.628\ Ca_2$$

The accompanying figure shows the percentage variations of the actual values from the theoretical values obtained from the above

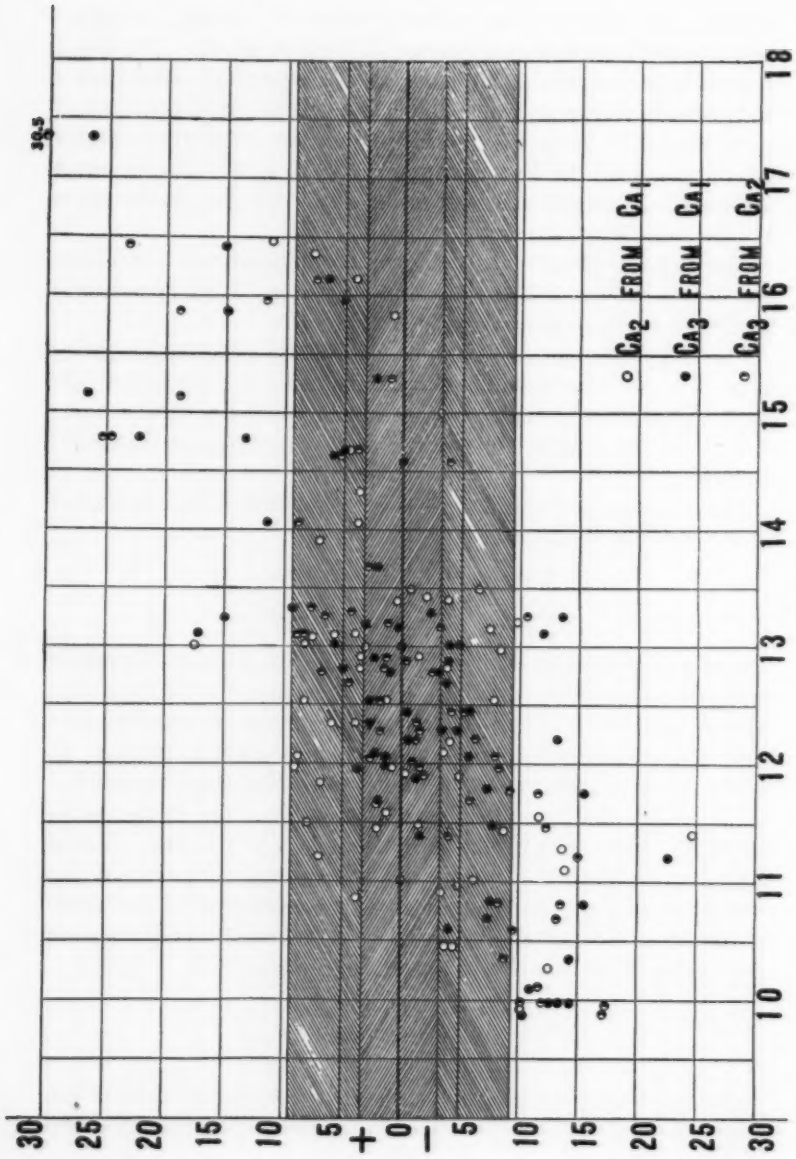
18.5
Ca2 FROM Ca1
Ca3 FROM Ca1
Ca3 FROM Ca2
30 25 20 15 10 5 + 0 − 5 10 15 20 25 30
10 11 12 13 14 15 16 17 18

FIG. 1

equations. The mean value of these figures is 12.73 mgm. for the calcium and 7.3 per cent for the deviation. The physiological significance of the individual variations is revealed by the points within and outside of the cross-hatch lines. The lines drawn at +9.5 and −9.5 per cent each represent the maximum numerical percentage difference that occurred in any of the duplicate analyses. All the points outside of these lines are therefore physiologically significant. The lines drawn at +3.25 and −3.25 per cent, respectively, were chosen arbitrarily because calculation of the analytical differences between duplicate samples showed that 86 per cent of the mean values of duplicate analyses had an error of 3.25 per cent or less. This means that 86 per cent of the points between the 3.25 per cent and the 9.5 per cent lines represent physiologically significant variations. In like manner 14 per cent of the points between the 0 line and the 3.25 per cent lines are significant. A summation of the significant variations of the actual from the theoretical values shows a total of 125 out of 180 or 69.4 per cent.

DISCUSSION OF RESULTS

As in the case of the inorganic phosphate, the mean value for blood calcium in dairy cattle cannot be determined with accuracy by random sampling. Significant fluctuations occur from day to day, not at present explainable, even when the samples are taken under presumably identical physiological conditions. Although there are fairly high coefficients of correlation of the consecutive samples in our data, yet nearly 30 per cent of the individual samples varied 10 per cent or more from the regression line. This represents a mean actual variation of over 1.25 mgm. of calcium per 100 cc. of blood plasma. The maximum actual variation encountered was 4.05 mgm. calcium per 100 cc. plasma.

The distribution of the points on the chart is not without significance. The similar chart for inorganic phosphate, given in our previous paper (1) shows no relation between the actual phosphate value and the direction of the deviations from the theoretical. In the case of calcium, however, it is evident that a relatively low concentration of calcium on the initial day of a three-day series

definitely predicts that the subsequent days' concentration will be *lower* than is demanded by the coefficient of correlation, whereas a relatively high concentration of calcium on the initial day of a three-day series definitely predicts that the subsequent days' concentration will be *higher* than is indicated by the coefficient of correlation. No logical explanation for this phenomenon has occurred to us. The distribution of the points on the chart is too definite, however, to be ignored. It is especially remarkable because the coefficient of correlation of the blood calcium on successive days is definitely positive.

CONCLUSIONS

The calcium content of the blood plasma of dairy cattle is subject to significant fluctuations of an undetermined cause, in spite of a high coefficient of correlation of the calcium content of the plasma on successive days.

When a three-day series of blood samples is secured from dairy cattle on successive days, any deviation of the plasma calcium from the theoretical value will be negative when the plasma calcium is relatively low, and will be positive when the plasma calcium is relatively high.

ACKNOWLEDGMENTS

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THE BIOMETRY OF CALCIUM AND INORGANIC PHOSPHORUS IN THE BLOOD PLASMA OF DAIRY CATTLE

APPLICATION OF RESULTS TO BONE MINERALIZATION*

L. S. PALMER, R. A. GORTNER AND RACHEL RUDE

Division of Agricultural Biochemistry, University of Minnesota, University Farm, St. Paul

The problem of the nature of the calcium compounds of blood has long attracted the interest of biochemists, physiologists, and clinicians. The problem has many applications to both normal and pathological conditions.

Rona and Takahashi (1) concluded that calcium occurs in blood serum in two forms only, as calcium bicarbonate which is completely diffusible, and as a calcium-protein compound, the calcium of which is non-diffusible. The possibility of calcium occurring in the blood as calcium phosphate was also considered by Rona and Takahashi but rejected on the following grounds. They pointed out that the only form of calcium phosphate that could exist at the hydrogen ion concentration of blood would be an insoluble one. They reasoned that this compound would therefore have to be present in suspension and would thus be non-diffusible, whereas they found that the inorganic phosphorus of the serum is entirely diffusible.

According to Rona and Takahashi the calcium bicarbonate concentration of blood is expressed by the relation

$$\frac{(Ca'')\ (HCO_3')}{(H')} = 350 \times 10^{-6}$$

From this it appears that at constant pH the calcium concentration of the blood varies, in part at least, inversely with the bicarbonate concentration.

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The solubility of calcium carbonate in blood has been considered extensively by Hastings, Murray, and Sendroy (2) and by Sendroy and Hastings (3) both from an experimental and from a thermodynamic standpoint. Their conclusion is that blood is not supersaturated with respect to calcum carbonate; it is probably undersaturated. Blood serum, in their studies, showed a stoichiometric solubility product for calcium carbonate of $10^{-6.40}$ compared with $10^{-7.40}$ for calcium carbonate in salt solutions of comparable ionic strength.

The relationship between calcium and protein in blood has been investigated by Salvesen and Linder (4), Marrack and Thacker (5), Loeb (6) (7), Loeb and Nichols (8) (9), Hastings, Murray, and Sendroy (2), and Peters and Eiserson (10). All of these studies support such a relationship. Salvesen and Linder found that decreases in serum calcium in non-uremic cases of Bright's disease without phosphate retention are paralleled by decreases in plasma protein. Marrack and Thacker investigated the dialysis of calcium from solutions of blood proteins and from blood serum and concluded that the calcium of body fluids is partly in the form of non-ionized protein compound, the formation of which accounts for the non-diffusible calcium of serum. Loeb, and Loeb and Nichols have studied the diffusibility of calcium from solutions of egg albumin, from serum globulin and from serum itself from the standpoint of the Donnan equilibrium theory and have concluded that essentially all the calcium is accounted for as ionized and non-ionized calcium-protein compounds. Hastings, Murray, and Sendroy have formulated the the equation, $[Ca] = 0.014\ [P] - 1.4$[1] to express the relation between the total calcium and protein concentration in serum. The equation was based on their own data and the data of Salvesen and Linder. Peters and Eiserson have modified this equation to include the inorganic phosphorus of the blood. Their equation is $Ca = -0.255\ P + 0.556$ protein $+ 7$[2]. It is not

[1] In this equation [Ca] = mM calcium per kilogram H_2O and [P] = gram protein per kilogram H_2O. The minus sign is an error according to Peters and Eiserson (10).

[2] In this equation the Ca and P are milligram per cent and protein is per cent.

surprising that attempts made by Peters and Eiserson to calculate the calcum of blood by means of this equaton led in some instances to errors of great magnitude. Casual inspection shows that the expression they have formulated has no biological significance when the calcium equals 7 mgm. per cent, because at this value the equation resolves itself into P = 0.218 protein. This relation is, to say the least, improbable. In fact the alignment chart given by Peters and Eiserson shows no such relation between blood phosphate and blood protein when calcium equals 7 mgm. per cent.

We have applied the Peters and Eiserson formula to several samples of normal cattle plasma with the results shown in table 1. The data show that the formula has no value for cattle blood.

TABLE 1

Deviation of calculated from observed blood plasma calcium in dairy cattle, using the formula of Peters and Eiserson

COW	CALCIUM FOUND	CALCIUM CALCULATED	DEVIATION OF CALCULATED FROM ACTUAL VALUE	
	mgm. per cent	*mgm. per cent*	*mgm. per cent*	*per cent*
66	7.70	8.99	+1.29	+16.8
82	9.35	9.40	+0.05	+ 0.5
E33	8.09	9.56	+1.47	+18.2
87	9.60	9.41	−0.19	− 2.0
60	7.70	10.14	+2.42	+31.4
21	10.23	9.95	+0.28	− 2.7

The samples were picked because they showed considerable range of calcium.

Although, as already stated, Rona and Takahashi rejected the probability of calcium existing in the blood as phosphate, there is indirect evidence which is generally accepted as indicating a close relationship between calcium and inorganic phosphate in the blood. Binger (11), and Tisdall (12) showed that the injection of phosphates reduces blood calcium and causes tetany. It should be pointed out, however, that although the results of Binger were attributed to the effect of the phosphate ion on blood calcium, Tisdall's results were interpreted as due to a changed sodium-calcium ratio. Disodium phosphate was injected in

Tisdall's experiments. In addition to this indirect evidence there exist the extensive thermodynamic studies of Holt, La Mer, and Chown (13), Holt (14), and Sendroy and Hastings (3) (15), on the solubility of calcium phosphates in biological fluids. The former group of investigators conclude that, "serum is normally *supersaturated* with tertiary calcium phosphate to the extent of more than 200 per cent." The experiments of the latter group of workers also support a condition of supersaturation of tri-calcium phosphate in blood. On the other hand, Shear and Kramer (16), and Shear, Washburn, and Kramer (17) hold that the calcium phosphate of serum is di-calcium phosphate and that normal serum is either very slightly *undersaturated* or just saturated with respect to this compound.

The occurrence of calcium phosphate in blood in the form of $CaHPO_4$ or $Ca_3(PO_4)_2$ is generally regarded as necessary in order to account for the mineralization of bone. Even though compounds of this character account for only a portion of the total calcium of the blood, it would appear probable that a large part of the inorganic phosphate would be involved. In this case definite biometric relations should be found to exist between the calcium and inorganic phosphate of the plasma or serum.

The biometry of calcium and inorganic phosphorus in the blood serum of rabbits has been studied by Harnes (18) (19). In a series of 80 samples, representing 80 animals received at the laboratory in groups of 10 during 8 consecutive months from October to May, the coefficient of correlation of P·Ca was

$$r_{P \cdot Ca} = -0.146 \pm 0.073$$

In a second series of 170 samples of the blood from 10 rabbits taken at weekly intervals for 17 weeks from October to May, the animals being kept under laboratory conditions, the coefficient of correlation of P·Ca was

$$r_{P \cdot Ca} = -0.124 \pm 0.051$$

In neither series is the result mathematically significant, inasmuch as the correlation in each case is less than three times the probable error. It is of interest to note, however, that the corre-

lation is a negative one, thereby lending some support to the fact that there is frequently a tendency for the inorganic phosphate to vary inversely with the calcium in blood serum or plasma.

EXPERIMENTAL

The normal variations in the calcium and inorganic phosphate of the blood plasma of dairy cattle, secured at this laboratory, have

TABLE 2

Mean, standard deviation, and coefficient of variation of calcium and inorganic phosphate in blood plasma of dairy cattle

	MEAN VALUE	STANDARD DEVIATION	COEFFICIENT OF VARIATION	NUMBER OF SAMPLES
	mgm.	*mgm.*	*per cent*	
Ca_1[1]	13.25	1.81	13.67	60
Ca_2	12.56	1.46	11.61	60
Ca_3	12.76	1.71	13.44	60
Ca	12.86	1.69	13.17	180
P_1	4.94	2.85	57.77	60
P_2	4.75	2.52	53.09	60
P_3	4.85	2.72	55.98	60
P	4.85	2.70	55.74	180

[1] The subscripts refer to the day in the three-day series.

TABLE 3

Coefficient of correlation and probable error of calcium and inorganic phosphate in blood plasma of dairy cattle

	COEFFICIENT OF CORRELATION	PROBABLE ERROR	NUMBER OF SAMPLES
$r_{Ca_1Ca_2}$	+0.762	±0.036	60
$r_{Ca_2Ca_3}$	+0.625	±0.053	60
$r_{Ca_1Ca_3}$	+0.628	±0.053	60
$r_{Ca\ Ca}$	+0.628	±0.031	180
$r_{P_1P_2}$	+0.970	±0.005	60
$r_{P_2P_3}$	+0.954	±0.008	60
$r_{P_1P_3}$	+0.931	±0.011	60
$r_{P\ P}$	+0.946	±0.005	180

recently been published (20) (21). The data were secured from samples of blood taken on 3 consecutive days at monthly intervals. Twenty-four different animals furnished the blood. Five of these had only 1 period of 3 consecutive days, 10 animals had 2 periods,

1 animal had 3 periods, and 8 animals had 4 periods. There were, therefore, 60 sets of three-day samples.

The previous papers give the complete data for the calcium and inorganic phosphate for the consecutive days. The mean values, standard deviations, and coefficients of variation are given in table 2. The coefficients of correlation, calculated by the method of Harris (22), and their probable errors are given in table 3.

The original data also presented an opportunity to study the coefficients of correlation of the calcium and inorganic phosphate, and thus obtain further light on the probable importance of calcium phosphate in the blood. The results of these calculations are as follows:

$$r_{Ca_1P_1} = +0.116 \pm 0.086 \quad (n = 60)$$
$$r_{Ca_2P_2} = -0.109 + 0.086 \quad (n = 60)$$
$$r_{Ca_3P_3} = +0.008 + 0.087 \quad (n = 60)$$
$$r_{CaP} = +0.022 \pm 0.050 \quad (n = 180)$$

These results show even less correlation between calcium and inorganic phosphate in the blood plasma of dairy cattle than was found for rabbits by Harnes (18) (19). In the rabbit blood the coefficients did approach but did not reach mathematical significance, *i.e.*, three times the probable error. In our calculations the summation correlation of Ca·P shows a probable error of nearly two and one-half times the correlation. The only conclusion that can be drawn from such a result is that the amount of calcium phosphate in the blood, at least of dairy cattle, is truly insignificant in relation to the other compounds of calcium and inorganic phosphate. We thus have mathematical proof for the conclusion of Rona and Takehashi (1) and of Loeb (6) (7), and Loeb and Nichols (8) (9) in so far as the occurrence of compounds of calcium and phosphate in the blood are concerned. It thus seems necessary to seek for an explanation of the mineralization of bone on other biochemical grounds than a mere precipitation of calcium phosphate from body fluid. Freudenberg and György (23) have proposed a theory which appears to deserve more recogniton that it has received. According to this theory

calcification of bone consists of the following stages: (*a*) A calcium-protein compound is formed between the collagen of the cartilage and the diffusible calcium ions from the blood; (*b*) HPO_4'' and HCO_3' ions from the blood react with the calcium bound by the collagen; (*c*) $Ca_3(PO_4)_2$ and $CaCO_3$ split off, releasing the calcium binding groups of the protein, so that the process may be repeated.

SUMMARY AND CONCLUSIONS

A high coefficient of correlation exists between the phosphate of the blood plasma of dairy cattle on successive days.

A fairly high coefficient of correlation exists between the calcium of the blood plasma of dairy cattle on successive days.

No correlation whatever exists between the calcium and inorganic phosphate in the blood plasma of dairy cattle.

No biologically significant amount of calcium phosphate occurs in the blood.

The mineralization of bone is not explainable on the basis of a mere precipitation of bone salts from body fluid.

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EFFECT OF THE ELECTROPURE PROCESS AND OF THE HOLDING METHOD OF TREATING MILK UPON BACTERIAL ENDOSPORES*

A. J. GELPI, JR. AND E. D. DEVEREUX

Department of Bacteriology, Michigan State College, East Lansing, Michigan

The Electropure process of milk "purification" has attracted some attention in recent years. The theory and mechanics of the Electropure process have been thoroughly described in the literature, and will, therefore, not be discussed in detail in this paper. Briefly, the process consists of passing a column, or rather a continuous stream of milk, through a narrow gauge rectangular chamber, the side walls of which consist of two especially prepared carbon electrodes. The resistance offered by the various electrolytes in the milk to the passage of electricity immediately causes the liberation of heat sufficient to effect a reduction of approximately 99 per cent in the bacterial count of the milk without apparently altering the quality of the milk.

During the course of some investigations carried on for the purpose of studying the types of microörganisms in raw market milk which seemed persistently to resist the Electropure process of milk "purification," it was oberved that strikingly few, if any, of the organisms which survived the process were spore-formers. As a result of this observation, series of tests were conducted in order to determine definitely whether or not the Electropure process actually destroyed spores, and, if so, to what extent.

In 1924 Carpenter et al (1) working with a culture of *Bacillus subtilis*, a microscopic examination of which revealed that the majority of the cells had developed spores, obtained approximately an 80 per cent reduction when 45 to 50 gallons of inoculated milk were subjected to electrical pasteurization.

The organisms used in our tests were all obtained from the stock cultures of the Michigan State College bacteriological

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laboratories. The cultures were grown for four to six weeks on agar slants at room temperature, and before using each culture was examined microscopically to determine the approximate extent of sporulation. All cultures showed from 80 to 90 per cent spores when used. Those cultures which were too dry to be put into suspension by the usual method of washing with saline solution, were ground in sterile mortars with sterile sand, then washed in salt solution and filtered through paper. Pure cultures of each organism were subjected both to the holding method of pasteurization and to the Electropure process according to the procedure outlined below.

The holding method was simulated as follows: 10 cc. lots of skimmed milk were placed in test tubes and rendered sterile by autoclaving at 10 pounds pressure for 20 minutes. Platings were made to check the sterility of the milk. Skimmed milk was used to avoid any interference from the fat layer in the test tubes. Each tube was then inoculated with about 0.1 cc. of the particular spore suspension to be tested. Immediately following inoculation, plate counts were made of each tube on standard agar plates using dilutions of 1:1000, 1:10,000, and 1:100,000. The tubes were then placed in an automatically controlled water bath at 62.8°C. and held at that temperature for 30 minutes from the time the milk in the tubes reached the desired temperature. At the end of the heating period the tubes were immediately transferred to an ice water bath and cooled to 10°C. Plate counts were then made of each tube, and the percentage reduction as a result of the treatment, was determined.

Each culture used in the above tests was then individually subjected to the Electropure process according to the following procedure. A two liter sample of skimmed milk previously rendered sterile by autoclaving for 20 minutes at 10 pounds pressure was heavily inoculated with saline suspension of spores (pure culture) of the organism to be tested. The milk used was carefully checked for sterility by running control plates. The flasks of milk were inoculated with such quantities of suspension that 1 cc. of the milk gave a plate count of 50,000 or over before treatment. After thoroughly shaking to insure an even distribution of

organisms, the inoculated milk was plated according to standard methods of milk analysis in order to obtain accurate initial counts. The milk was then subjected to the Electropure treatment at a temperature of 71°C. The milk entered the machine at room temperature and reached the desired temperature by the time it had travelled two-thirds of the way through the chamber. Thirty-five to forty seconds were required for the milk to pass through the machine, during 10 to 14 of which it was subjected

TABLE 1

Percentage reduction in spore count due to pasteurization

CULTURE	HOLDING PROCESS (62.8°C.—30 MINUTES)			ELECTROPURE PROCESS (71°C.)		
	Initial count	Final count	Percentage reduction	Initial count	Final count	Percentage reduction
First trial						
B. anthracis	2,170,000	2,110,000	2.7	2,400,000	4,200	99.5
B. megatherium	4,810,000	4,812,000	0	2,600,000	740,000	71.5
B. subtilis	490,000	550,000	0	2,100,000	360,000	82.0
B. mycoides	678,000	682,000	0	3,000,000	5,000	99.9
B. mesentericus	1,162,000	1,140,000	1.7	7,800,000	150,000	98.0
Second trial						
B. anthracis	947,000	944,000	0.3	1,850,000	4,700	99.7
B. megatherium	3,720,000	3,780,000	0	14,500,000	570,000	90.6
B. subtilis	1,970,000	1,960,000	0.5	1,140,000	13,000	98.0
B. mycoides	1,620,000	1,400,000	13.0	4,200,000	2,000	99.9
B. mesentericus	2,180,000	2,160,000	0.9	5,200,000	310,000	94.0

to a temperature of 71°C. A sample was drawn in a sterile flask (about 75 to 100 cc.) after the rate of flow and temperature had been properly adjusted and were constant. Immediately upon drawing, the sample was cooled to below 20°C. and plated as above. The results of these tests (plate counts and percentage reduction as a result of treatment for each process) are shown in table 1.

SUMMARY

Series of laboratory tests were conducted with some common spore-forming organisms in order to compare their relative re-

sistance to the Electropure and holding methods of pasteurization. The cultures were from six to eight weeks old when used, and microscopic examinations showed them to contain from 80 to 90 per cent spores. The results obtained in these tests, as shown in table 1 are markedly in favor of the Electropure process. Particular attention is drawn to those results obtained with *Bacillus anthracis* which is known to be an extremely resistant organism. The percentage reduction in the case of this organism ranged from 0.3 to 2.7 in the holding method, and from 99.5 to 99.7 in the Electropure process. The results of the tests with *B. subtilis*, *B. mycoides*, *B. mesentericus*, and *B. megatherium* are equally striking.

Concluding from the above results concerning spore destruction the Electropure process at 71°C. with momentary holding proved to be superior to pasteurization at 62.8°C. for 30 minutes, in these laboratory tests.

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MEASURING THE INFLUENCE OF HORMONE AND MECHANICAL STIMULATION BY MEANS OF A NON-FECUND LACTATION*

R. B. BECKER

Florida Agricultural Experiment Station, Gainesville, Florida

AND

P. C. McGILLIARD

Oklahoma Agricultural Experiment Station, Stillwater, Oklahoma

Milk secretion is caused by two factors (1), namely, an unusually marked internal or hormone stimulus, and an external mechanical or nervous stimulation produced by the act of milking. The latter has less influence than the former. Lane-Claypon and Starling (2) established a relationship between development of the foetus and of the mammary gland. Likewise, d'Errico (3) found that the blood of a pregnant bitch exerted an inhibitory influence on milk secretion when injected into the veins of a lactating bitch.

Pregnancy and parturition have a marked influence on the development and activity of the mammary gland, and provide the initial stimulus (1) for milk production, but are not essential (4) in connection with a limited milk yield. Literature relative to milk secretion by virgin and non-fecund females, and males of several species, has been reviewed extensively by Hammond (4), Hill (5), Marshall (6), Monvoisin (7) and Velich (8).

PRESENTATION OF DATA

An unusual instance of non-fecund lactation occurred with the Holstein-Friesian cow, Rosa Segis Johanna 446097, in the Oklahoma Agricultural Experiment Station dairy herd. This cow had previously completed yearly semi-official records of 18,375.7 pounds of milk, 3.45 per cent fat, 635.13 pounds of butterfat as a junior two-year-old, and of 24,313.7 pounds of milk, 3.55 per cent fat, 864.36 pounds of butterfat as a senior three-year-old. She

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was placed on semi-official test again, but was withdrawn from test after an operation to remove a nail that had pierced the reticulum and diaphragm near the heart. A subsequent operation was attempted on an infected Fallopian tube. Following the latter operation, her ovaries ceased to function, voice assumed a masculine tone, crest began to develop, coccygeal vertebrae showed an inclination to rise as in certain cases of sterility in cows, and she exhibited the desire to mount other cows in the herd at every opportunity. She went dry on August 17, 1925, and was excessively fat. Her blood was negative to the agglutination test for the *Bacillus abortus* of Bang (*Brucella abortus*).

Rectal examinations were made, and the ovaries massaged regularly by Dr. E. E. Harnden, veterinarian on the staff of the Oklahoma Agricultural and Mechanical College. A cyst on the right ovary was ruptured on January 14, 1926, and after the usual interval of four days thereafter the cow came into heat, but was not bred. She showed oestrum again at a three weeks interval on February 8, 1926, but was not deemed in condition to be bred.

Two hundred cubic centimeters of oestrual hormone solution were injected in 10 cc. doses between January 15 and February 11, 1926, without any effect being noted that could be attributed to the injections. The influence of this solution, according to Dr. H. S. Murphey (9), is that "The only known hormone action of the corpus luteum is to produce a substance which affects the peristalsis of the tubular part of the genitalia. You can find plenty of statements in the literature about this structure being a gland of internal secretion, but there is no proof which will stand experimental investigation."

It is not known whether the ovarian extracts exerted any influence, but the fact remains that the cow gradually resumed feminine characteristics of voice and behavior. She was irregular in the oestrus cycle. After being pronounced in good health by the veterinarian, she was bred on May 3 and 15, 1926, but did not conceive to either service. She was bred five more times at various intervals and finally conceived on July 11, 1927. A 92-pound bull calf was born on April 8, 1928 following a 273-day gestation period.

The most interesting fact concerning this cow, is that after being dry from August 17, 1925, and not pregnant, her udder began to develop in June, 1926. One quarter was found to be infected. In treating it, this quarter was milked out daily. Stimulation of the udder and teats brought the cow into milk. From July 1,

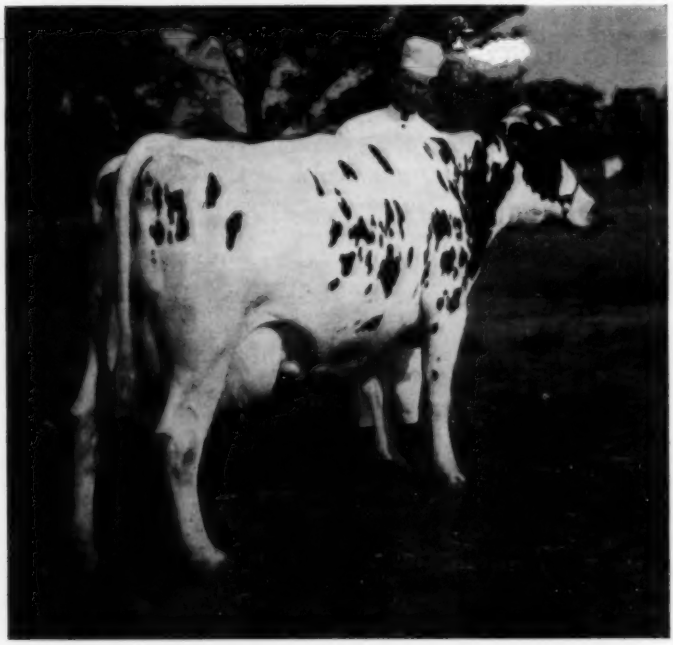

FIG. 1. ROSA SEGIS JOHANNA 446097 AS A FOUR YEAR OLD

1926 to August 3, 1927, she was milked twice daily. Since conception occurred only 24 days prior to completion of this non-fecund lactation, no decline in milk flow due to pregnancy was anticipated (4, 10, 11).

As a measure of the factors governing milk yield, the non-fecund lactation is compared with two previous and one subsequent lactations following normal gestation periods. Although the cow was

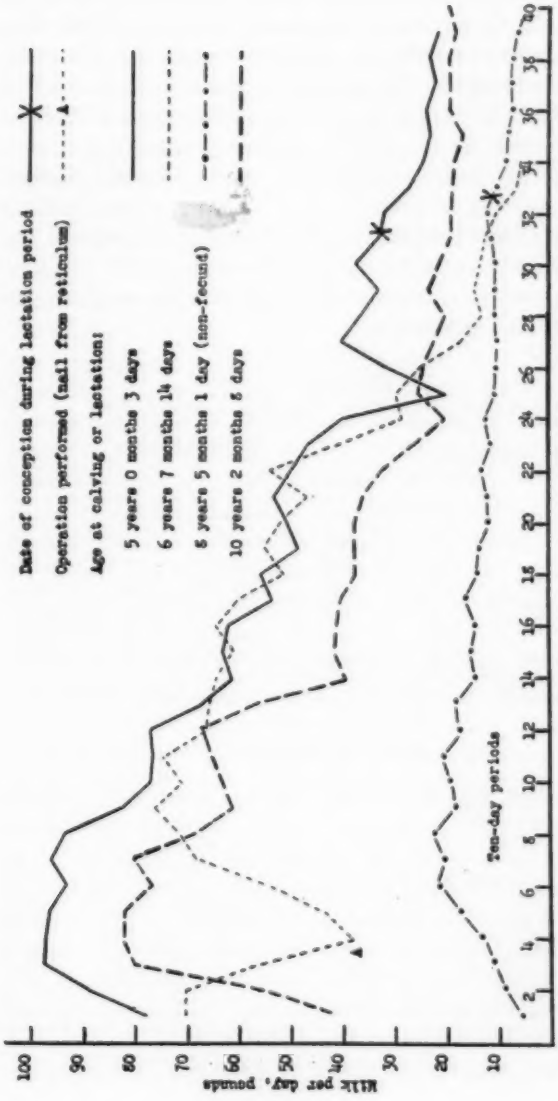
Date of conception during lactation period
Operation performed (nail from reticulum)
Age at calving or lactation:
5 years 0 months 3 days
6 years 7 months 14 days
8 years 5 months 1 day (non-fecund)
10 years 2 months 8 days
Milk per day, pounds
Ten-day periods

FIG. 2. NON-FECUND AND NORMAL LACTATION CURVES OF ROSA SEGIS JOHANNA 446097, COMPUTED BY TEN-DAY PERIODS, INCLUDING DATE OF CALVING AS THE FIRST DAY

milked twice daily during the non-fecund lactation, during the early parts of the three normal lactations she was milked three times daily in order to care for the high milk yields. Milk yields in the highest month of the normal lactations, were 2,987.5, 2,287.0 and 2,552.1 pounds respectively, as compared with 611.8 pounds in the highest month of the non-fecund lactation.

The lactation curves are tabulated by ten-day periods, including the day of calving as the first day. These are presented in figure 2. Production records, length of gestation periods, and age of the cow at time of calving, are presented in table 1. Feed and care were given the cow commensurate with her requirements for maintenance and production.

TABLE 1

Lactation and gestation records of Rosa Segis Johanna 449097

AGE AT CALVING			PRECEDING GESTATION	PREGNANT DURING LACTATION	LACTATION RECORDS			
					Length	Milk	Test	Fat
yrs.	*mos.*	*days*	*days*	*days*	*days*	*pounds*	*per cent*	*pounds*
2	1	21	*	*	365	18,375.7	3.45	635.13
3	9	27	*	*	365	24,313.7	3.55	864.36
5	0	3	274	115	423	21,612.6	3.66	790.97
6	7	14	282	0	338	15,014.6	3.99	599.65
8	5	1	0	24	399	5,199.1	3.41	177.16
10	2	8	273	0	523	17,024.8	3.30	562.11

* Prior to purchase by the Oklahoma Agricultural Experiment Station.

DISCUSSION OF RESULTS

Normal lactation curves measure the combined effect of the factors that govern milk production. Previously, we (12) showed maximum daily milk yield and persistency of lactation to be hereditary characters involved in total milk yield during normal lactations. The graphs in figure 2, show the effect of the mechanical and hormone influences on maximum daily milk yield, rate of decline in milk production, length of lactation, and time of conception of the mature Holstein-Friesian cow Rose Segis Johanna during three normal and one non-fecund lactations.

The normal lactation curves shown in the graph have the char-

acteristic maximum daily milk yield which measure the combined influences of the internal stimulus released at parturition, and of the external mechanical stimulus arising from the act of milking. The non-fecund lactation curve shows practically no influence of internal stimulus, but measures wholly the effect of the external mechanical stimulus upon milk flow. Gestation was not advanced sufficiently to exert an influence (4, 10, 11) on the two lactations involved.

When the normal and non-fecund lactation curves are superimposed, the *difference* between them measures largely the effect upon milk flow, of the internal stimulus resulting from gestation and released at parturition. Since the cow was milked three times daily to care for the extra milk yield in the three normal lactations, allowance must be made for the influence of the third milking daily. This has been variously estimated (13, 14, 15) as increasing the milk flow from 6.0 to 21.4 per cent under ordinary herd conditions, and as high as 48 to 72 per cent (10, 16, 17) under official testing conditions. (Official testing conditions involve a plane of nutrition above the cow's requirements, milking 3 or 4 times daily, housing in a box stall, and other items of management that permit maximum inherited capacity for production to be attained.)

It is seen from the super-imposed graphs, that the stimulus arising from reproduction had its greatest influence in the first few weeks after parturition. This internal secretion gradually diminished either in amount or effect until it appeared that the last few weeks of lactation were prolonged by external stimulation alone (10).

Observation of the shape of the curve of non-fecund lactation shows that in this instance maximum daily milk yield was attained later in the lactation period, and that at the peak it was only 21 to 28 per cent as high as in the normal lactations. Also the rate of decline in milk flow was less rapid, due to absence of a diminishing influence of an internal stimulus or hormone. Duration of the non-fecund lactation compared favorably with that of three normal lactations by the same cow.

SUMMARY

The review of literature and limited data obtained from an unusual case of non-fecund lactation in a Holstein-Friesian cow, indicate that the internal stimulus produced by pregnancy and released at parturition, was the largest factor controlling maximum daily milk yield. The more rapid decline in milk flow in normal lactations than in the non-fecund lactation, is attributed to a gradually diminishing internal or hormone influence arising from reproduction. Maximum daily milk yield was attained later in lactation when influenced mainly by external mechanical stimulation from the act of milking. This internal stimulus originating from reproduction, was not necessarily a factor in determining persistency of milk flow, as indicated by duration of the lactation period. At the peak of production, total daily milk yield following normal reproduction, was from $3\frac{1}{2}$ to 5 times as great as when milk flow was induced by mechanical stimulation alone. A small part of the difference is attributable to the fact that the cow was milked thrice daily in the normal lactations.

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THE CHEMICAL ANALYSIS OF BUTTER

The recognized value of the directions for operating the Babcock test and of the specifications for Babcock test glassware promulgated by the committee on this method for the American Dairy Science Association prompted an extension of this type of activity. The methods are considered primarily from the viewpoint of accuracy and standardization of testing procedures within the industry. The work will be of greatest value if those concerned with the use of these tests will express their views to the chairmen of the various committees so that methods can be adjusted to give most general satisfaction. The following report constitutes the second procedure recommended through the committees on chemical methods. The method is adapted to the complete analysis of butter in the laboratory rather than for a rapid churn test.

The two general methods of making a complete analysis of butter in a creamery are: the procedure of the Association of Official Agricultural Chemists (A. O. A. C.), which is used by the Federal Government and State Chemists; and the Kohman test, which is simpler of operation and which better satisfies the creamerymen.

At present, only a few butter manufacturing plants are regularly making either daily or weekly analyses of butter formilkfat, water, salt, and casein. Most creameries make churn tests for water and salt and the milkfat is obtained by difference, after making an allowance for casein. The time is at hand when all creameries ought to know the complete analysis of their butter and a sufficient number of complete analyses should be made to make certain that the milkfat content is correct as secured by difference. This practice is followed at the present time in the better class of plants.

Recently Clarke (1) reported that during July and the first ten days of August, 14.2 per cent of the butter in interstate shipments through Chicago was adulterated. Probably most of this butter was not willfully manufactured with a low milkfat or a

high moisture content. Undoubtedly some of it was illegal butter just because of carelessness in not testing it or on account of poor testing equipment and methods.

Both the Kohman and A. O. A. C. methods have a high degree of accuracy, when properly executed. A comparison of them was made by Guthrie (2). Among a large number of data presented by him, the table (table 1) using Kohman's own figures,

TABLE 1

Differences in Duplicates

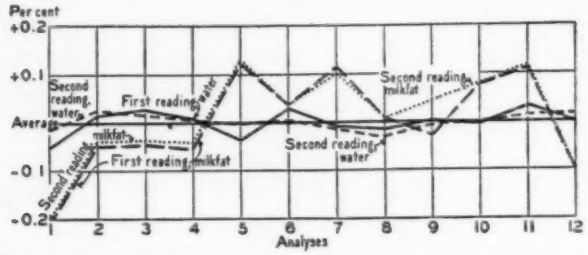
LOT NUMBER	A.O. A. C. TEST		KOHMAN TEST	
	Water	Milkfat	Water	Milkfat
	per cent	*per cent*	*per cent*	*per cent*
1	0.02	0.09	0.030	0.040
2	0.07	0.11	0.020	0.010
3	0.03	0.08	0.028	0.020
4	0.04	0.14	0.047	0.065
5	0.01	0.02	0.014	0.036
6	0.06	0.01	0.060	0.060
7	0.07	0.03	0.010	0.056
8	0.03	0.00	0.020	0.066
9	0.02	0.02	0.020	0.071
10	0.02	0.03	0.021	0.123
Average.....	0.037	0.053	0.027	0.0547

In the water analyses the average difference between the duplicate samples was 0.010 per cent in favor of the Kohman method. The milkfat determinations showed a preference in accuracy of 0.0017 per cent for the A. O. A. C. method.

and the two succeeding graphs indicate a close comparison of these two methods of analyzing butter.

In order to determine the accuracy of the modified Kohman method, when the weighings were made under a watch glass, a set of twelve portions was taken from a single sample. Approximately 10 grams of butter was put in an aluminum beaker, after which it was immediately covered with a watch glass and the weight was taken. The modified Kohman method was then followed. The first reading of the milkfat in this series, as noted in graph 1, showed an average variation of 0.113 $\pm$ 0.007 per cent, with a range from 0.002 to 0.337 per cent. The moisture in

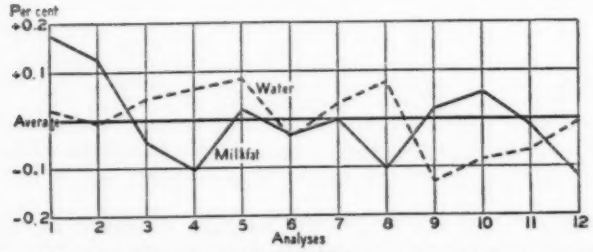
this reading had an average variation of 0.031 ± 0.002 per cent, with a range from 0.001 to 0.087 per cent. These portions were dried for an additional fifteen minutes, and were then cooled and



First reading:												
Water	16.568	16.638	16.650	16.633	16.586	16.653	16.613	16.607	16.624	16.620	16.655	16.634
Milkfat	80.166	80.328	80.337	80.333	80.503	80.410	80.476	80.366	80.331	80.454	80.492	80.290
Second reading:												
Water	16.628	16.666	16.656	16.653	16.639	16.654	16.631	16.615	16.643	16.645	16.659	16.660
Milkfat	80.163	80.328	80.334	80.323	80.500	80.417	80.493	80.376	80.353	80.459	80.492	80.288

Per cent

GRAPH 1. VARIATIONS WITHIN A SINGLE SAMPLE OF BUTTER
(Twelve analyses by the modified Kohman method)



Water	16.952	16.050	16.614	16.638	16.635	16.586	16.603	16.644	16.434	16.484	16.506	16.553
Milkfat	80.132	80.079	79.910	79.854	79.978	79.915	79.964	79.848	79.974	80.012	79.943	79.843

Per cent

GRAPH 2. VARIATIONS WITHIN A SINGLE SAMPLE OF BUTTER
(Twelve analyses by the A. O. A. C. method)

again weighed. The second reading, as may be seen in graph 1, had a mean variation in milkfat of 0.117 ± 0.007 per cent, with a range from 0.001 to 0.337 per cent. The water had an average difference, out of the 66 possible variations, of 0.018 ± 0.001 per cent, with a range from 0.001 to 0.051 per cent.

Inasmuch as the modified Kohman method gave very satisfactory results when the butter was weighed in a closed cup, it seemed advisable to again see what the results would be in a second trial of the A. O. A. C. procedure. The butter was weighed in a covered cup and then analyzed. The data in graph 2 show that the mean variation in milkfat was 0.104 ± 0.006 per cent, with a range from 0.004 to 0.289 per cent. The average difference in the water was 0.081 ± 0.004 per cent, with a range from 0.006 to 0.221 per cent.

The manner in which Guthrie (2) executed the modified Kohman method is outlined under "Operation," page 387. The Association of Official Agricultural Chemists (A. O. A. C.) procedure was as follows:

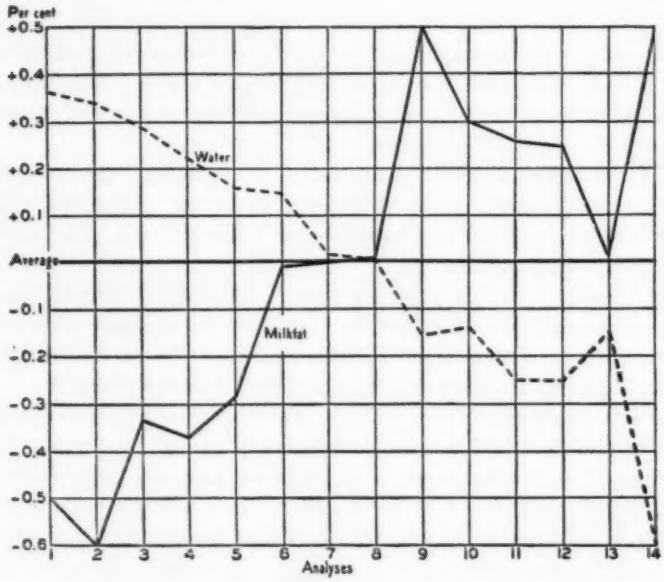
A sample of butter weighing from 1.5 to 2.5 grams was placed in a flat-bottomed dish having a surface of at least 20 square centimeters. It was then dried in a hot-water oven and weighed hourly during the process until the weight was constant.

When determining the milkfat, the whole sample was washed into a Gooch crucible with petroleum ether as a solvent. The washing was continued until the filtrate was free from milkfat. The Gooch crucible then was placed in the hot-water oven to dry. When hourly weighings showed constant weight, the amount of the milkfat was obtained by deduction as follows: water 14.5027 per cent + casein and salt 3.2104 per cent = 17.7131 per cent; 100 per cent − 17.7131 per cent = 82.2869 per cent, the milkfat content.

It should be noted that these studies were made in a well equipped laboratory with balances accurate to 0.1 mgm., and where it was easily possible to place approximately ten grams of butter in the beaker and to cover it immediately to prevent evaporation of moisture, and then to ascertain the exact weight.

Conditions, however, in the creamery are quite different, for the balances are standardized to weigh exactly 10 grams. It is necessary, therefore, to work carefully and rapidly in making the weighing without much loss of moisture while the butter is on the scales. In the average creamery, where the whole procedure is not as accurately conducted as in a well organized laboratory,

it is probably advisable to use simplified equipment such as the Torsion balance where 10 grams of butter are used and where the readings, in moisture, are obtained directly in percentage. When



GRAPH 3. VARIATIONS WITHIN A SINGLE SAMPLE OF BUTTER

(Fourteen analyses by the modified Kohman method)

This graph shows the variations that existed between the different analyses in a sample of butter. All of the analyses were made after the butter had been carefully prepared in a 500-cc. wide-mouthed bottle. The graph does not include the variations due to sampling from churn, tub, roll, or print. The weighings were made on a balance that read to 0.1 mgm., which naturally would show less variation than the scales in use in the average creamery. The butter was weighed in an open aluminum cup; this is the practice in most creameries, and is not satisfactory.

running the tests in this manner, the operator of a butter factory should not expect to do closer weighing than is shown in graph 3.

In the studies by Guthrie an electric hot plate was employed. Most creameries, at present, can be supplied with this convenience. But if they do not have electricity nor gas, an alcohol

lamp may be used to evaporate the moisture. When a flame is used, gasoline can not be evaporated over it, because gasoline is very inflammable. Other means of drying in this case are necessary.

When exactly 10 grams of butter are weighed into the beaker, as in case of the usual creamery practice, sufficient moisture evaporates during the weighing process to make a marked error. Graph 3 shows that the variation in water was from approximately -0.6 per cent to about $+0.36$ per cent. In milkfat the range was from approximately -0.6 per cent to $+0.5$ per cent. These wide variations were due to the evaporation that took place during weighing. In contrast to this graph, it should be observed in graphs 1 and 2 that the variations are small. The reason for this is that the analyses reported in these two graphs were run under a better laboratory technique, where finer balances were employed, and where approximately 10 grams of butter were placed in the beaker and then weighed under a watch glass. The average creamery operator should expect to do closer weighing than is shown in graph 3, if he uses the equipment listed below, if he puts approximately 10 grams of butter in the cup under a watch glass, and if he is painstaking in his technique.

THE KOHMAN METHOD OF ANALYZING BUTTER

So long as the modified Kohman method of analyzing butter is more simple of operation than the A. O. A. C. procedure, it is being given preference for the operator of a creamery. And in order to particularly assist the creameryman, who is a layman in matters pertaining to chemical analysis, a complete list of equipment and supplies is hereby given, and a discussion of the operation of the test follows.

Equipment and supplies

A large spatula and a small spatula. The larger one may be made of hardwood, about $\frac{1}{16}$ by $\frac{1}{2}$ by 10 inches in dimensions. The smaller one should be of thin metal. A pocket knife will suffice.

A sample jar, in which the butter is thoroughly stirred to a

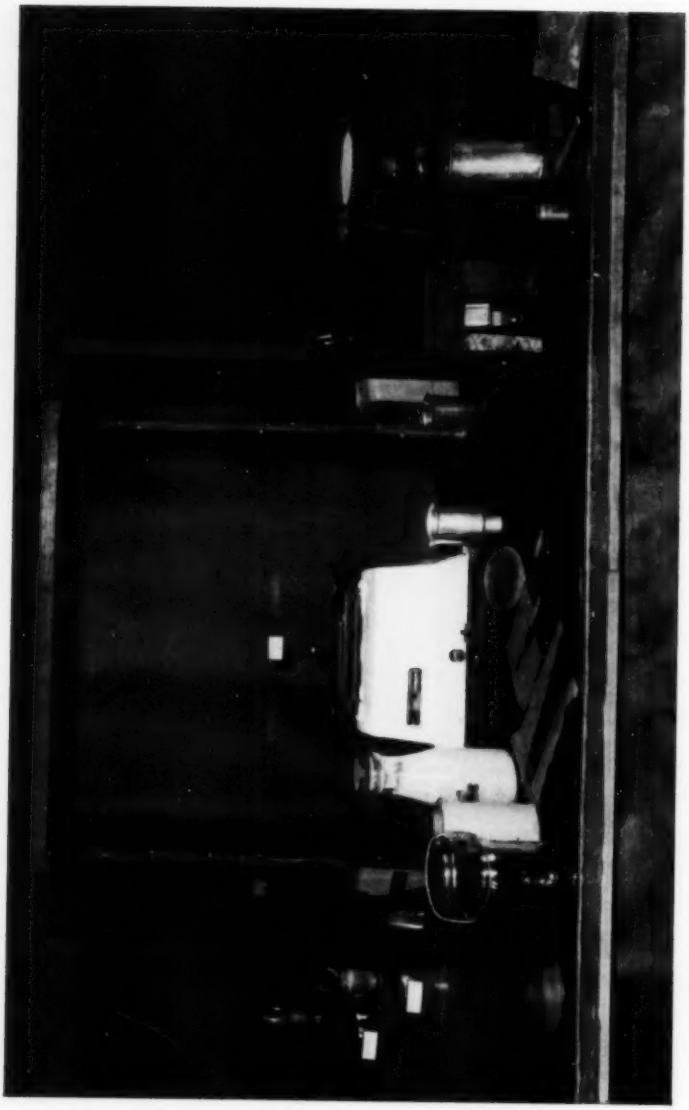


FIG. 1. EQUIPMENT AND SUPPLIES FOR THE MODIFIED KOHMAN METHOD OF ANALYZING BUTTER

creamy consistency, which may be a pint milk bottle fitted with a number 9 rubber stopper. For an alternative method of preparing a butter sample, a pint fruit jar that is fitted with a rubber ring may be employed. In this second procedure (3) the butter is melted and then it is vigorously shaken during the cooling process.

A metal beaker or dish approximately 2 inches in diameter and 3 inches high. A small pair of forceps to handle the weights and to shove the riders back and forth on the beams. A larger pair of forceps, for lifting the beaker, was unintentionally left out of figure 1.

A set of weights weighing from 1 mgm. to 100 grams, that costs about $5. A torsion balance valued at about $40, which may be kept in a home-made cabinet. A tripod with an asbestos mat. An alcohol lamp. A box of matches. A supply of gasoline, such as is found in all automobile filling stations. A thin metal shelf. A cooling surface such as an old flatiron or a smooth building stone, or concrete.

A roll of tissue. A rubber-tipped glass rod or soft-wood stirrer. A supply of silver nitrate. A burette. A bottle of potassium chromate. A pipette. A graduated cylinder or a flask. A white cup and a stirring rod with a color scale is convenient.

Operation

1. Obtain the sample. When sampling from the churn the hardwood spatula is satisfactory. The extraneous moisture may be eliminated by scraping off the surface of the butter in the churn, over a small area, and immediately obtaining a portion of approximately 10 to 15 grams. For every-day churn analysis, a sample should be made up of 10 or 12 portions. This butter should be placed in a jar that has been dried over a steam pipe or in a similar way, for at least several hours. The type of jar will depend on the method employed in preparing the sample. When the butter is thoroughly mixed by stirring, a pint glass milk bottle properly fitted with a rubber stopper is satisfactory, for the neck is long and sloping. In a milk bottle, small portions of

butter can not stick in inaccessible places, as is true of bottles with square shoulders.

When the sample is taken from the package and not from the churn, a portion should be obtained from each tub or printer box. This should give a representative sample of the finished product of a single churning. If the sample is obtained during the packing process a portion of soft butter may be obtained from each package before it is finished off. When the sample is obtained from hard butter, a single trierful taken by boring diagonally from the top to the bottom will suffice. About an inch of butter from each end of the trier, in this case, should be eliminated.

2. Balance the metal beaker or dish, placing the metal disc or a watch glass over the top. The doors of the cabinet, during weighing, should be at right angles with the case, so that the balances may not be affected by drafts of air.

3. The sample of butter should now be prepared for weighing. When the butter is brought to a creamy consistency by stirring, it should be warmed in water at a temperature from 100° to 110°F. until the milkfat has almost reached the melted stage. In case the test does not have to be completed quickly, the butter may be softened at room temperatures. If care has been exercised in warming the sample, so that it is not too soft, it will not be necessary to cool it during the latter part of the preparation. Thus, time may be saved. When a sample of butter is being prepared, it should be tightly closed, with the exception of when it is being stirred. During the weighing the jar should be made tight as quickly as the butter has been taken out.

4. By the employment of the large hardwood spatula, place approximately 10 grams of butter in the beaker or dish. The small metal spatula or pocket knife should be used here, to scrape the butter from the large one into the dish. The butter should be landed on the bottom of the dish, and not smeared over the sides. The toilet tissue is convenient to wipe the butter from the spatula, the knife, and other apparatus.

Time should not be taken to weigh exactly 10 grams of butter into the dish, for there will be too much evaporation of moisture

before the weight is obtained. The metal disc or watch glass should be placed on the dish as quickly as the butter is delivered into it. This precaution in weighing makes necessary the use of the block of weights that weigh to 0.01 gram. In case the buttermaker wishes only a close approximation of the composition of his butter, he can save time by weighing out exactly 10 grams of butter. Then the percentage moisture can be read directly on the beams of the scale. Thus, he is saved the time of computing the percentage. To illustrate the obtaining of the amount of butter in the former case, let us say that the beaker weighed 25.48 grams, and that the beaker and the butter amounted to 35.12 grams. The difference is the weight of the butter, $35.12 - 25.48 = 9.64$ grams of butter.

5. By the use of the large forceps transfer the dish from the balances to the asbestos pad, in case a flame of gas or alcohol is used to drive off the moisture, or to the hot-plate if electricity is the heating medium. During the drying process, it is desirable to use an asbestos pad over a flame, for otherwise carbon is likely to deposit on the dish and affect the weight. The employment of rather low temperatures, such as 275° to 300°F. is essential, for at higher temperatures the butter is likely to spatter out of the dish. At this time it is wise to shake the samples sufficiently often to completely break up the casein blanket. When this casein surface is not properly broken, particles of it will float away later, on the gasoline. This causes an error in the analysis.

6. As quickly as the milkfat has reached a golden-brown color, the dish should be placed, with the forceps, on a smooth and clean iron or stone surface to cool. The forceps should be employed in preference to the hands, for the grease or moisture of the hands might change the weight of the dish. Iron or stone, which have an extremely high capacity of heat conduction, will rapidly cool the beaker and its contents. This cooling will be accomplished in 5 minutes, which is about the same as the time consumed in reducing the temperature over an electric air-fan or by cooling in cold water. When the last method is followed, there is danger of not removing all the moisture from the dish,

even though it is wiped with a dry cloth. It is usually advisable to place a weight on the dish or beaker when it is cooling, so that its contact with the iron will be intimate. The cooling iron should be free from dust, grease, and the like, in order that the weight of the dish will not be affected.

7. The cooled beaker and its contents weighed 33.63 grams. 35.13 grams (weight of beaker and butter in step 4) − 33.63 grams = 1.49 grams (weight of evaporated moisture). The percentage of moisture is $\frac{1.49 \text{ grams} \times 100}{9.64} = 15.45$.

8. Fill the cup to within about $\frac{3}{4}$ inch from the top with gasoline, and thoroughly stir it with either a rubber-tipped glass rod or a softwood spatula. The purpose of the gasoline is to dissolve the milkfat. After it has stood approximately three minutes, the salt and casein will have settled to the bottom. Then the gasoline with its load of milkfat may be carefully poured into the drain. The beaker must be handled gently, so that the salt and casein will remain at the bottom. If little flakes of casein float on the surface of the gasoline, they are an indication that the casein film was not properly broken by agitation during the process of the evaporation of the moisture, as pointed out in step 5.

9. Again fill the beaker with gasoline and thoroughly stir the mixture. After standing another three minutes this gasoline may be poured off. Where it is wise to be economical in the use of gasoline, this second washing may be saved, to be used in the first extraction in the succeeding analysis. At this stage about 2 cc. of gasoline will remain in the beaker.

10. If an electric hot plate is employed, the beaker or dish with the remaining salt and curd may be dried by placing it immediately on the plate. In case the evaporation must be accomplished over a flame of gas or alcohol, the remaining gasoline should be largely dried off over a steam pipe or a radiator, for gasoline is too inflammable to be placed directly over a flame at this stage of the analysis. The thin metal shelf shown in figure 1, may be placed over the steam line for convenience in holding the beaker at this period of the process. The completion of the

drying process may be accomplished over a low flame when an asbestos pad is employed. The contents of the beaker when evaporation is completed, should be largely in the form of a dry powder.

11. Cool the beaker and its contents and then weigh. The calculation of the amount of milkfat is done as follows: 33.63 grams (weight before milkfat was extracted) − 26.19 grams (weight after milkfat was extracted) = 7.84 grams of milkfat.

The percentage of milkfat is $\frac{7.84 \text{ grams} \times 100}{9.64 \text{ grams (weight of butter)}} = 81.32$.

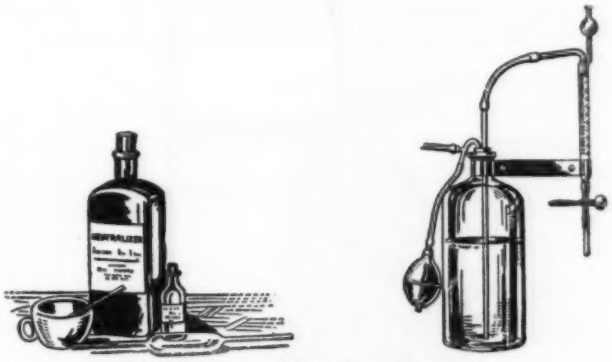


FIG. 2. THE TROY SALT TEST

12. In preparation for determining the amount of salt, transfer all of the contents of the beaker to the 300 cc. flask, in case of the Troy salt test, or a 250 cc. flask if the Nafis salt test is employed. This transfer may be accomplished by filling the beaker approximately three-fourths full of lukewarm water and stirring the mixture with a rubber-tipped glass rod or a softwood spatula. Then after pouring the contents of the beaker into the flask, the procedure should be repeated until all of the salt and curd have been transferred to the flask. Now the flask should be filled to the 300 cc. or 250 cc. mark with warm water.

13. Shake the flask sufficiently long for the salt to be uniformly

distributed throughout the solution. Now measure out 17.6 cc. of the salt solution when the Troy salt test is being used, or 25 cc. if the Nafis test is being employed; and put it into a white cup for titration.

14. After putting 2 or 3 drops of indicator (potassium chromate) into the salt solution in the white cup, titrate into it the silver

FIG. 3. THE NAFIS SALT TEST

nitrate from the burette to a brownish-red color. In the Troy salt test the silver nitrate is a tenth normal solution, and in the Nafis salt test it is a special strength but in both cases the dilutions are calculated so that the cubic centimeters of silver nitrate

used is equal to the percentage of salt. In this analysis it was 2.5 per cent.

15. The following procedure is followed in calculating the percentage of curd. The sum of the moisture, 15.45 per cent, milkfat 81.32 per cent, and salt 2.5 per cent, is 99.37 per cent. 100 per cent − 99.37 per cent = 0.63 per cent curd.

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Sub-committee on Chemical Analysis of Butter:

E. S. GUTHRIE, *Chairman.*
V. C. MANHART,
D. H. NELSON,
H. B. ELLENBERGER.

Committee on Chemical Methods:

A. C. DAHLBERG, *Chairman.*

SUGGESTED METHODS FOR THE MICROBIOLOGICAL ANALYSIS OF BUTTER

FOREWORD

The American Dairy Science Association, through its committee on bacteriological methods, is contemplating the formulation of a complete set of microbiological analytical procedures useful in controlling the quality of dairy products. This committee expects to act largely through sub-committees appointed from men in the Association who have had experience with the bacteriological analysis of various dairy products. Obviously, the formulation of such methods should not be left to the arbitrary decision of a committee, but should be the result of suggestions and criticisms coming from all interested parties, whether or not they are members of the American Dairy Science Association. The committee, therefore, wishes to serve as a center about which methods satisfactory to the largest number may be evolved.

The rapid development in the use of laboratory methods in the control of butter manufacture has made it necessary to study the various methods now used in order to adopt uniform procedures (which at the present time do not exist) for the control of microörganisms in butter. These methods will be revised later in accordance with the suggestions received and the judgment of the committee, before they are finally adopted by the American Dairy Science Association and included in the report on bacteriological methods of analyzing dairy products.

THE AGAR PLATE METHOD FOR THE MICROBIOLOGICAL ANALYSIS OF BUTTER

Sterilization of glassware. Same as outlined in the latest edition of Standard Methods of Milk Analysis, American Public Health Association, 370 Seventh Avenue, New York City.

Sterilization of equipment used in sampling. Metal triers, spatulas, spoons to be wrapped in paper (Imported Kraft wrap-

ping paper usually withstands sterilization temperatures without charring), or enclosed in metal containers and sterilized in the same manner as glassware, or in the autoclave at 15 pounds steam pressure for 30 minutes.

Wooden tongue depressors serve as satisfactorily as spatulas. They may be wrapped in paper or enclosed in a metal container and sterilized by autoclaving, or by hot air.

Method of sampling. 1. Butter in the churn. After the butter has been worked and is ready to be removed from the churn, samples for microbiological analysis should be taken by means of a sterile trier, taking three samples of about 1 ounce each, one sample from the center of the churn, the other two from the respective ends.[1] The butter should then be placed in sterile screw top sample jars.

2. Tubs or packages. About 1 ounce of butter should be removed from two different parts of the tub or package with a sterile trier, the plugs of butter to be not less than 2 inches in length and to include the surface portion. The butter should be transferred from the trier to the sterile screw top sample jar with the aid of a sterile spatula or spoon.

3. Print butter. Because of difficulty in obtaining the same amount of exposed surface of butter on one, one-half and quarter pound prints, the method of sampling print butter should be by means of a trier. By using a small trier and plugging the end of the print, a plug of 3 to 4 inches may be obtained. Such a plug will weigh about half an ounce. The butter should be transferred from the trier to a sterile screw top sample jar with the aid of a sterile spatula or spoon.

Care of samples. All samples should be placed in cracked ice immediately after sampling or placed in a refrigerator where the temperature does not exceed 4°C., and plated as soon as possible.

[1] Under commercial conditions where large numbers of samples must be taken daily, it may be impractical to employ a sterile trier for each sample. If a polished trier is wiped thoroughly after each sample with tissue paper until the surface is highly polished and then plunged into the butter to be sampled at least twice before the sample is taken, satisfactory results can be obtained with a single trier.

Where samples are procured from a grading station, remote from the laboratory, the sample should be stored at −10°C., or lower until shipped.

For shipping, the samples should be placed in a precooled iceless ice cream shipping container and packed carefully so as to prevent breakage. Upon receipt of the samples at the laboratory they should be plated as quickly as possible and record should be made as to the number of days elapsed between sampling and plating.

Preparation of samples for plating. Place the sample jar containing the butter to be tested in a water bath between 40 and 45°C., and shake until the butter is of a creamy consistency. The time required for the sample to reach a creamy consistency should not exceed 15 minutes. Plating should follow immediately.

Preparation of dilutions. With a previously warmed 1 cc. pipette,[2] the melted butter should be drawn up into the pipette, care being taken to have as little butter as possible on the outside of the pipette. To remove as much butter as possible from the pipette, blow hard through the pipette into the dilution blank which has been previously warmed to a temperature of 40 to 45°C. Rinse the pipette by sucking the dilution water into the pipette.

DETERMINATION OF YEASTS AND MOLDS

Media:

1. Bacto Dehydrated Whey Agar.
2. Bacto Dehydrated Malt Agar.
3. Whey Agar made according to one of the following formulae:
   I. Coagulation with Acid.
      1. Use fresh, skimmed milk.
      2. Place skimmed milk in water bath and hold at a temperature of 35°C. while adding dilute hydrochloric acid to precipitate casein. (To prepare the dilute acid, add 1 part of hydrochloric acid containing 31.45 per cent hydrochloric acid, sp. gr. of 1.16 or 20 degrees Baumé, to 8 parts

[2] The specifications for the pipette to correspond with the specifications of milk pipettes as given in Standard Methods of Milk Analysis.

of distilled water. To prepare 1.16 hydrochloric acid from stock concentrated acid, dilute 29.3 cc. with 5.2 cc. of distilled water. To prepare the acid for precipitating, dilute the resulting mixture with 276 cc. of distilled water.)

3. Bring whey to a pH of 4.6 (Colorimetric or potentiometric method may be used.)
4. Filter whey through cheese cloth to remove curd.
5. Place whey in autoclave for 15 minutes at 15 pounds pressure.
6. Filter through absorbent cotton.
7. Neutralize filtrate with N/1 NaOH to a pH of 6.8 to 7.0.
8. Add 0.5 per cent peptone and then autoclave for 15 minutes at 15 pounds.
9. Filter through filter paper.
10. Add 1.2–1.5 per cent agar to whey broth and heat in steam bath to melt agar.
11. Filter through cotton if necessary.

II. Coagulation with Rennet.

1. Use fresh skimmed milk.
2. Heat to 38° to 40°C. Add about 2 cc. rennet extract per liter and allow the milk to curdle firmly. Break up the curd thoroughly so that it will contract rapidly, heat to about 55°C. to aid this contraction, and separate the whey by straining through cheese cloth.
3. Weigh out peptone equal to 0.5 per cent of the whey and agar equal to 1.5 per cent of the whey. Add these to one-half the whey, weigh and then heat until the added ingredients are dissolved and restore the water lost. Combine the two portions of whey and adjust the pH to 6.4 to 6.8 and the temperature to about 55°C.
4. Add egg (in the proportion of 1 whole egg to 2 or 3 liters of medium) and distribute thoroughly by stirring and then pouring the medium from one container to another.
5. Flask and autoclave at 15 pounds pressure; the time of heating is dependent on the size of the flasks.
6. The medium may be filtered through cotton and tubed or bottled at once, or it may be stocked in the flasks and melted, filtered, and tubed, or bottled as needed.

The media should be sterilized in definite quantities, preferably 100 cc. by autoclaving at 15 pounds steam pressure for 20 minutes.

Acidulating the medium. Adjust the reaction of the warm sterile agar to a pH of 3.5 with sterile lactic or tartaric acid solution before pouring. Never heat the agar after the addition of the acid, as heating at an acidity of pH 3.5 may destroy the jellying properties of the agar.

The amount of lactic or tartaric acid to be used varies according to the buffering qualities of the medium. Five per cent acid solution is recommended.

Colorimetric or potentiometric methods should be used to determine the amount of acid necessary to bring the pH of the medium to pH 3.5.

By using the indicator brom phenol blue, a yellow green color is obtained at pH 3.5.

As an index as to the amount of 5 per cent lactic acid of 85 per cent purity required, Hood has compiled the following table:

MEDIUM	APPROXIMATE AMOUNTS OF 5 PER CENT LACTIC ACID SOLUTION TO 100 CC. OF MEDIA	FINAL pH
	cc.	
Bacto Dehydrated Whey Agar	5.5	3.5
Bacto Dehydrated Malt Agar	2.5	3.5
Whey Agar (homemade)	10.0	3.5

Table taken from Studies on Moulds and Yeasts in Creamery Butter Pamphlet 92, New Series, Department of Agriculture, Dominion of Canada, 1928.

Dilutions. Dilutions suggested for determining the yeast and mold content of butter are 1, 0.1 and 0.01 of a cc. Higher dilutions may be made if desired.

Plating. About ten cc. of acidulated melted agar cooled to a temperature of 40 to 45°C. should be poured into each petri dish after the introduction of the sample and thoroughly mixed with the sample by a rotary motion.

The plating should be completed as rapidly as possible.

After the medium has thoroughly hardened, invert the petri dishes and place in incubator.

Incubation. The plates should be incubated at either 21° or 25°C. for 5 days.

The plates should be examined after two or three days incubation to determine the amount of mold growth and, if the molds are developing in large numbers, a count should be made at this time and a recount on the fifth day.

Counting. Count the number of mold and yeast colonies without the aid of a counting lens.

Reporting results. The number of yeast and mold colonies per cubic centimeter should be reported separately, and together, as a total count. The kind of medium used should be reported. Where media are clarified with egg this fact should be stated.

TOTAL BACTERIAL COUNT

Medium. The following media are suggested for the determination of the bacterial count in butter:

1. Bacto Peptonized Milk Agar.
2. Whey Agar—pH 6.6 to 6.8.
3. Beef Infusion Agar, containing 1 per cent lactose.

For the preparation of Beef Infusion, see General and Pathogenic Bacteriology, Moore and Hagen, P. 9, Ginn & Company, New York, 1925. The above media should be adjusted to a pH of 6.8.

Dilutions and plating. According to Standard Methods of Milk Analysis, using dilution blanks at a temperature of 40° to 45°C.

Incubation. (*a*). Five days at 21°C. or (*b*) four days at 21°C. and 1 day at 37°C.

Counting. According to Standard Methods of Milk Analysis.

Expression of results. The results as to the total number of colonies per cubic centimeter of butter should be expressed as total count. The kind of medium used and the temperature of incubation should be reported.

PROTEIN DIGESTING BACTERIA

There are two methods suggested for the determination of protein digesting bacteria in butter. They are:

1. By picking colonies from the plates that have been used to determine the total number of bacteria in butter and inoculating

these colonies into tubes of sterile milk containing either brom-cresol-purple or litmus.

For the preparation of the indicator brom-cresol-purple, see Journal of Agricultural Research, Vol. 10, No. 3, p. 103–111, July, 1917.

All colonies should be picked from the plate or contiguous colonies from a given sector of the plate.

Incubation of tubes should be for 5 days at 21°C.

Expression of results. Results are to be expressed as the proportion of colonies picked that show proteolysis in milk.

2. By using a medium that contains casein and noting the proteolysis as it occurs on the plate.

The following media are suggested:

A. Milk agar:

	grams
a. Agar	15.0
Water	900.0
b. Skimmilk	100.0

Flask separately in portions for plating; sterilize, cool to 45°C. and pour plates. The medium should have a pH of 6.6–6.8.

After incubation, flood the plate with dilute acid (1 part concentrated hydrochloric acid to 10 parts of water). If clearing remains, it denotes true hydrolysis of the casein, if not, it is a weak acid clearing.

B. Casein agar, (Frazier, Wm. C. and Rupp, P.: Studies on the proteolytic bacteria of milk. Journal of Bacteriology, Vol. 16, 57–78, 1928).

Incubation. Five days at 21°C.

Expression of results. The number of proteolytic colonies per cubic centimeter of butter should be reported with a record of the medium used.

AN ADAPTATION OF THE FROST LITTLE PLATE METHOD FOR THE RAPID QUANTITATIVE DETERMINATION OF MOLDS AND YEASTS IN CREAMY BUTTER

Mr. C. K. Johns, of the Division of Bacteriology, Dominion Department of Agriculture, Ottawa, has worked out the follow-

ing modification of the Frost Little Plate method for determining the mold and yeast count of butter:

Reference: A rapid method for determining the mould and yeast count of butter. Sci. Agric., 8, 353–369, 1928.

Sampling. Same as outlined for the agar plate method.

Preparation of samples. Same as outlined for the agar plate method.

Care of sample. Same as outlined for the agar plate method.

Preparation of dilutions. Ten cubic centimeter portion withdrawn with a sterile warmed pipette, taking care to remove the butter from the outside of the pipette as completely as possible. Contents then introduced into dilution bottle containing 40 cc. water at 45°C. Rinse the pipette by sucking up dilution water.

Preparation of slide. An area 2 by 4 cm. is marked upon a clean microscopic slide, using a wax pencil and guide. Slide is then placed on a warm table maintained at 45°C.

Medium. Same as outlined for yeasts and molds in the agar plate method. When ready to prepare micro-plate, place 4 drops of medium upon slide.

Making micro-plate. After thorough agitation of the dilution a portion is quickly withdrawn with a sterile 1 cc. pipette graduated in tenths. Two-tenths of a cubic centimeter is placed upon the slide, mixed with the medium by means of a sterile needle, and spread uniformly over the measured area of the slide. The warm table prevents the agar from solidifying before the operation is completed. The micro-plate is then removed to a cold level surface and protected from air contamination while hardening.

Incubation. The hardened micro-plates are placed in a moist chamber, which contains sufficient moisture to prevent the drying out of the agar during incubation. The moist chamber containing the micro-plates is then removed to the incubator. Incubation at 25°C. for 15 hours gives the best results. If a shorter period must be used, the temperature may be raised a few degrees; if a longer period is more convenient, the temperature may be slightly lowered.

Staining. At the conclusion of the incubation period the micro-plates are removed and dried on a metal sheet placed over boiling

water. This operation should take about 5 to 10 minutes; if dried too fast or left drying too long the film has a tendency to crack. When dry the micro-plates are placed in Coplin jars containing thionin stain made up as follows:

Thionin[3]	1 gram
Carbolic acid	2.5 grams
Distilled water	400 cc.

Mix, filter and add 5 per cent glacial acetic acid.

Slides should be stained for 3 minutes, then carefully washed in clean water and dried.

Adjustment of microscope. Microscope should be adjusted so that the area of the field under the low power objective will be approximately 2 sq. mm. For details regarding calibration of microscope see latest Edition of Standard Methods of Milk Analysis of American Public Health Association.

Counting colonies. After a preliminary survey to observe the evenness of distribution of the colonies, 20 representative fields are counted. If no colonies are encountered in 20 fields, another 20 may be counted. If the mold and yeast count runs over 50 colonies per field the counting of 10 fields is sufficient. Yeast and mold counts are made in duplicate and averaged. The count per cubic centimeter is then calculated according to the following formula:

$$\frac{A}{B} \times X \times Y \times Z = N$$

where A = number of colonies observed, B = number of fields counted, X = reciprocal of dilution, Y = reciprocal of quantity plated, Z = microscopic factor, N = number of colonies of molds (or yeasts) per cubic centimeter. *E.g.*, if the microscopic factor (area of micro-plate area of field) is 400, and the total count on 20 fields gives 4 molds and 30 yeast colonies we would have

$$\frac{4}{20} \times 5 \times 5 \times 400 = 2{,}000 \text{ mold colonies per c.c.}$$
$$\frac{30}{20} \times 5 \times 5 \times 400 = 15{,}000 \text{ yeast colonies per c.c.}$$

Where the microscopic field is adjusted to 2 square millimeters and 0.2 centimeter of a 1/5 dilution plated, as in the example

[3] Thionin, synonym Lauth's Violet, not Thionin Blue.

above, calculation is simplified by averaging the number of molds (or yeasts) per field and multiplying by 10,000 ($X \times Y \times Z$).

The 1/5 dilution will enable counts to be obtained up as high as 50,000 molds or 1,000,000 yeasts per cubic centimeter of butter.

The counts of molds obtained by the micro-plate method are on the average twice as great as those obtained by the standard plate method; each individual colony resulting from the germination of a single spore can be counted, while with the standard plate method two or more spores may germinate and grow together to form a single macroscopic colony.

Remarks. Mr. Johns requests that more work be done on this method in order to determine its value under different conditions. The method is designed primarily to enable the detection of highly contaminated butter in a shorter period of time than is possible by the standard plate method.

A DIRECT MICROSCOPIC METHOD OF EXAMINING BUTTER FOR MICROÖRGANISMS

Reference: A Microscopic Method for Examining Butter for Microörganisms, G. L. A. Ruehle, Michigan Acad. Sci. 21st Report, p. 123–125, 1919.

Method of sampling, preparation of samples and care of samples of butter are the same as outlined for the agar plate method.

The steps in making a direct microscopic examination of butter are as follows:

1. The butter is weighed out, using aseptic technic; 2 grams of butter are used.

2. The butter is transferred to a clean separatory funnel previously warmed to 40°C. The separatory funnel should be carefully washed and treated with dichromate cleaning solution before being used again.

3. Two cubic centimeters of warm sterile skimmed milk in which the precipitate has been allowed to settle, for at least one week after sterilization, is added to the butter, and the mixture rotated until the butter has melted and mixed with the milk.

4. One hundred cubic centimeters of a fat solvent (ether) is added and the funnel gently agitated to dissolve the fat, exercising

care to avoid foam formation. The fat solvent should also be added a little at a time to prevent resolidification of the fat.

5. After dissolving the fat, the contents of the funnel are allowed to separate and the aqueous portion drawn off into a thoroughly clean beaker.

6. One fiftieth (1/50) of a cubic centimeter (after thorough mixing) is spread out with a needle on a clean glass slide so that it covers an area of one square centimeter.

7. The smears are now treated in the same manner as milk smears for direct microscopic counting. The procedure is outlined in the latest edition of Standard Methods of Milk Analysis.

One-fiftieth of a cubic centimeter of the aqueous portion represents approximately one hundredth of a gram of butter.

A MICROSCOPIC METHOD FOR THE DETERMINATION OF YEASTS AND OIDIA IN CREAM AND BUTTER

Reference: H. W. Redfield. The determination of yeasts and oidia in cream and butter. Jour. of Dairy Science, 5, 14–21, 1922.

By means of a small butter trier, 3 or 4 cores from different portions of the butter sample are transferred to a 6 by 1 inch test tube, usually about half filling the tube. The butter is melted at 45°C. and allowed to stand at temperatures between 45° and 40°C. until the fat is separated in a clean layer with the mixture of curd and whey or brine at the bottom of the tube. With a sterile 1 cc. pipette the curd and whey are thoroughly mixed by drawing the liquid back and forth in the pipette. Then 1 cc. of this mixture is transferred to a clean watch glass care being taken to wipe thoroughly all the fat from the outside of the pipette with a clean towel before discharging the pipette into the watch glass. A Petri dish may be used but is not as satisfactory. With a Breed pipette 0.01 cc. of the mixture is transferred to a microscopic slide, carefully spread over an area of 1 sq. cm., 2 sq. cm., or 4 sq. cm., depending on the quality of the butter, diluting if necessary with distilled water in order to obtain uniform smears. After drying in the air, extracting with xylol, and fixing with 95 per cent alcohol and the slide is stained and dried in the air.

Counting is done with a combination of lens and draw tube length to give a factor of 500,000. The diameter of the field should be 0.16 mm. to give a factor of 500,000. In enumerating yeasts and oidia each cell, whether separate or as an element in a chain or budding colony, is counted. The number of fields to be counted depends upon the number of organisms present. It is recommended that 100 fields be counted except in very high count products.

CONCLUSION

The views expressed in this report are those of a committee appointed by the American Dairy Science Association. As such they are printed for the general criticism of other members of the A. D. S. A., and other interested parties.

Separate copies of this report, and of the Ice Cream Report previously issued may be secured at cost from the chairman of the Committee on Bacteriological Methods.

Sub-Committee on Microbiological Methods of Examining Butter:
E. H. PARFITT, *Chairman,*
E. G. HOOD,
B. W. HAMMER,
H. MACY.

Committee on Bacteriological Methods:
R. S. BREED, *Chairman.*

THE EFFECT OF INITIAL COOLING TEMPERATURE ON GELATIN IN THE AGING OF THE ICE CREAM MIX*

K. E. WRIGHT

Department of Dairy Industry, Massachusetts Agricultural Experiment Station, Amherst, Massachusetts

Gelatin has generally been used in ice cream to improve the texture by partially preventing the formation of ice crystals. The partial occlusion of water by the gelatin also results in a product which is less watery in consistency. However, the swelling property of gelatin and its behavior in ice cream are not thoroughly understood. Obviously, therefore, if more were known about gelatin, its effect on ice cream could be more readily explained.

The fact is generally known that gelatin will swell to many times its volume in water by physical combination, although the extent of true hydration of gelatin as shown by Theones (1), Adair (2), and Moran (3) is less commonly known. Except for Theones's results they determined that each gram of gelatin combines with less than its own weight of water or 1.86, 0.6, and 0.53 gram of water respectively. Even the latter figure is considered equivalent to 1500 molecules of water per molecule of gelatin, according to Moran.

The above figures for physical and chemical combination appear very high and therefore seem to preclude further study of such constants until those factors are investigated which often cause unexplainable behavior in gelatin solutions. This is necessarily so when one considers that notable increases in the viscosity of an aged ice cream mix are not always proportional to the small quantity of gelatin used. Thus an understanding of gel structure can result only following a consideration of the factors dealing with the formation of a new phase as related to a protein colloid.

The process of aging has long been regarded as a colloidal

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phenomenon. Although there is no reference in the literature which satisfactorily explains the process of aging, the results of the process are quite apparent. Aging is normally accomplished by rapidly cooling the mix to about 40°F. after homogenization. By holding at this temperature for twenty-four hours, an increase in viscosity is obtained which approaches the maximum obtainable under the condition stated. Although the degree of viscosity thus attained has been looked upon somewhat as a measure of the completeness of aging, no direct relation has been established between this viscosity and the improved whipping property of the mix and the smoother texture of the finished product. These latter effects are, however, variable to the extent of insignificance.

Further evidence of the lack of practical knowledge regarding aging may be noted by comparing the following methods of treating the mix. Turnbow and Raffetto (4) state that the mix aged at 32°F., not to exceed 36°F., is of better quality and has more viscosity than one aged at higher temperatures. The other extreme is evident in a study by Lucas and Scott (5) in which they state that a hot gelatin solution was added when the mix had cooled to 70°F. Such a method is different in that complete sudden cooling is not possible. From the above it is quite apparent that a better understanding of aging is needed.

Only mixes containing gelatin are appreciably influenced by aging. Thus, a better conception of the factors bearing on the aging phenomenon should be obtained with the aid of a good theory of gelatin structure. Until comparatively recently the structure of gelatin was regarded as a fibrous network by Bogue (6) and others. However, Ostwald (7) summarized theories on "gel" formation and elucidated the theory that "gel" structure is a secondary fusion of enlarged particles. The same idea is substantiated in a recent study by Krishnamurti (8), a student of Donnan, on the scattering of light in colloidal sols and gels. In this study the intensity depends on distinct changes in the shape of the particles. There was no rapid change observed either during gel formation or as a result of agitation of a gel. This indicates that the particles were spherical, because any other shape would show variations when the axis of the particle is moved with

respect to a plane of light. This is especially marked in the case of a rod shape particle. Furthermore, these facts substantiate the work of Bradford (9) who obtained microphotographs of gelatin crystals which he calls spherites. The above conception would indicate, therefore, that the real or basic viscosity of ice cream mix is due largely to the number and size of the particles.

In the aging of ice cream certain properties affecting the size of gelatin particles have been overlooked which, theoretically, have a bearing on the mix. Gelatin is influenced by the phenomenon of hysteresis which is characterized by a difference between the solidifying point and the melting point. These points are affected

TABLE 1

The effect of basic viscosity on the overrun of the ice cream mix, as due to the influence of the initial aging temperature on gelatin content

	BATCH 1	BATCH 2	BATCH 3	BATCH 4	BATCH 5
Initial aging temperature, degrees F....	46	70	81	100	107
Viscosity, degrees M.....	53	184	190	210	185
Mix temperature at the tenth minute, degrees F.....	24.5	24.7	24.6	24.4	24.4
Overrun at tenth minute, per cent.....	99	95	92	90	90

by the rate of cooling, the significance of which has been brought out by Arisz (10). He observed that a gelatin solution at 68°F. solidified much sooner than a similar solution at 35°F. Arisz interprets his finding as a condition in which the equilibrium necessary to a change of state could take place at the higher temperature more readily than at the low temperature. This is so because the heat influences the molecular activity necessary to the formation of nuclei and particles which in turn determine the basic viscosity due to the size and number of particles.

Theoretically, the observation is extremely significant with respect to aging the ice cream mix inasmuch as the mix is supercooled with respect to the gelatin content. Hence, the development of viscosity with aging is the result of the tendency of the gelatin to attain equilibrium essential to a change of state. The purpose of this study will therefore be an attempt to determine the significance of the initial cooling of the mix and its relation to aging, its effect on viscosity, and on the texture of ice cream.

EXPERIMENTAL METHODS

The mixes, varying in fat from 10 to 15 per cent and in serum solids from 9 to 10 per cent, contained 15 per cent sugar and 0.35 per cent gelatin (250 Bloom test) which was dispersed in cold skimmilk and cream. The sugar and skimmilk powder were then mixed and added. All mixes were pasteurized at 149° to 151°F. for thirty minutes. Homogenization followed at a pressure of 2500 pounds. The mixes were rapidly cooled over a surface cooler to the temperatures subsequently indicated and then were allowed to cool without agitation for twenty-four hours in a 40° cold room.

Preliminary viscosity trials with 200 cc. quantities indicated that the viscosity results with ice cream mix were far more comparable if the plastic structure as distinguished from basic viscosity by Leighton and Williams (11) was broken down by agitation with a high speed stirrer at about 65°F. until the viscosity readings were constant when observed at 68°F. Two minutes was found to be a sufficient period of agitation. DePew (12) describes a similar method for destroying the apparent viscosity. Disregard for apparent viscosity nullifies precise methods of calibrating a viscosimeter.

The viscosity was determined after twenty-four hours with a MacMichael viscosimeter using a No. 30 wire with the disc bob. The cup was rotated 19.1 r.p.m. Readings were taken when the temperature in the cup reached 68°F. All readings are stated in degrees M. After aging, the mixes were frozen in a forty-quart brine freezer when freezing data were required. A power driven hand freezer was satisfactory when accurate freezing data were not required. The fat and total solids were determined by the Mojonnier method.

EXPERIMENTAL RESULTS

The general effect of initial aging temperature on the viscosity of a solution containing gelatin is shown in figure 1. The data are plotted for viscosity obtained by allowing 200 cc. quantities of skimmilk containing 0.5 per cent gelatin to cool slowly from the

temperatures indicated to 40°F. Likewise every mix showed similar increases in viscosity depending on the initial aging temperature except that mixes without gelatin did not develop extra viscosity when initially aged at a high temperature.

Table 1 is a record of data obtained by slowly cooling 45-pound batches of mix without agitation from the temperatures indicated to 40°F. The table also shows the effect of viscosity on the percentage volume increase or overrun attained in 10 minutes. The figures show that the mix temperatures are practically constant.

The photograph, figure 2, shows the appearance of the melting samples at various intervals of time in a series of samples frozen

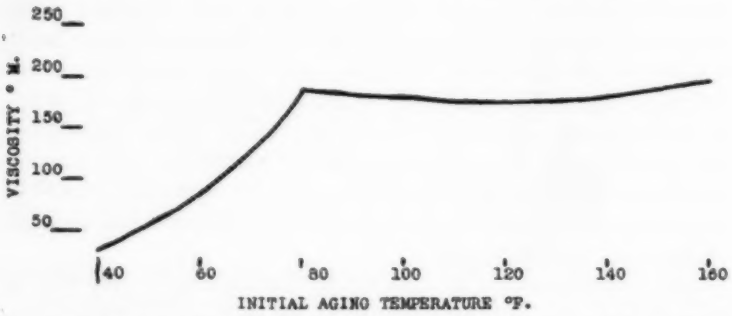

FIG. 1. EFFECT OF INITIAL AGING TEMPERATURES ON VISCOSITY OF 0.5 PER CENT GELATIN IN SKIMMILK

from batches initially aged at the temperatures indicated. While numbers 1, 2, and 3 show very definite progression in the rapidity of melting, numbers 4, 5, and 6 appear to be more constant in character of melting which tends to conform with the corresponding viscosity results. The melting samples of all temperature series studied showed the same general characteristics both in rate and manner of melt which progressed from smooth to rough in the direction of the high temperature.

The results in the foregoing figures and table are quite typical for a large number of mixes with respect to the trend of the curve. All the curves are characterized by an abrupt rise to approxi-

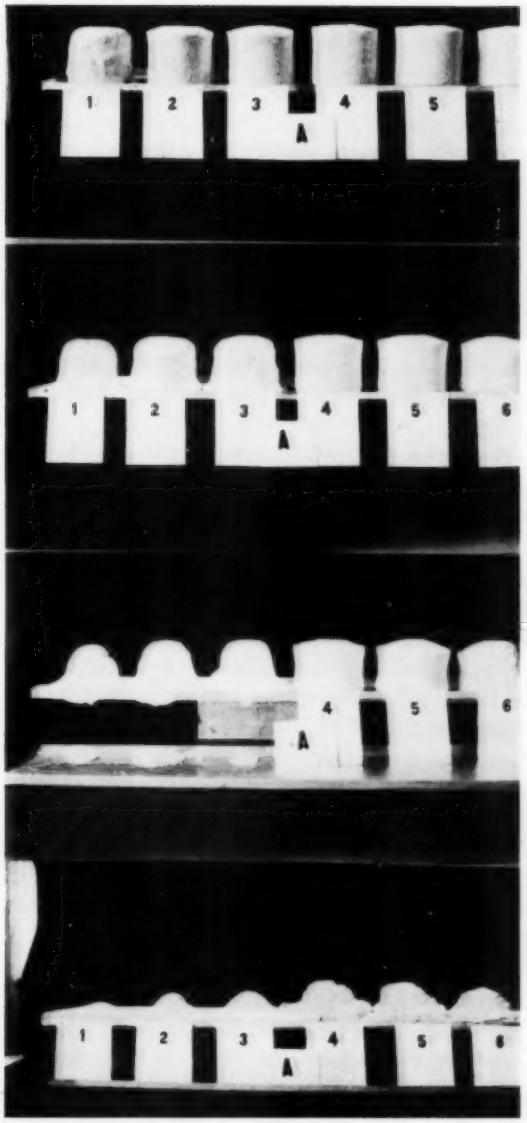

FIG. 2. THE APPEARANCE OF MELTING SAMPLES AT VARIOUS INTERVALS OF TIME

Sample number	1	2	3	4	5	6
Initial aging temperature	40	50	60	85	95	105
Viscosity, degrees M	100	120	130	260	230	231

mately 80°F. This rise varied from 50 to 400 per cent greater than the 40°F. viscosity result. Many of the series developed maximum viscosity at 80°F. while a few increased slightly in the case of the 100°F. initially cooled mix. Above this initial temperature, all mixes showed a decrease in viscosity with the exception of a few within the limit of experimental error. The rise in viscosity was accompanied by improvement in texture in the finished product.

The initial cooling temperature was not noted beyond the first observation because the agitation required to obtain a uniform temperature of a given portion would hasten cooling and possibly disturb gelation.

A more logical procedure is now being followed in the continuation of this study. Portions of a mix are held at the various temperatures for specific periods of time and are then rapidly cooled to 40°F. For example, maximum viscosity was developed in less than three hours by holding at 80°F. This method makes it possible to obtain a more definite idea of temperature effects as well as temperatures less favorable to bacteria.

In regard to bacteria, their growth and acidity development were considered to be very minor factors in comparison with the fundamental kinetic activity concerned in this study. Hence, bacterial counts and acidity data were not closely observed. The above viewpoint is taken from several different angles, including the fact that in at least one instance over 100 per cent increase in viscosity was noted between samples initially aged at 35° and 50°F. respectively.

DISCUSSION OF RESULTS

The characteristic rise in viscosity accompanying conditions of higher initial aging temperatures appears to be in perfect agreement with the principles of Arisz; namely, a condition permitting equilibrium of energy necessary in a change of state from a liquid to solid. This change and its relation to viscosity may be more readily understood with a consideration of Freundlich's (13) discussion on "The Kinetics of the Formation of a New Phase."

By interpreting the results obtained in the light of the above, the fact appears that the low viscosity of a mix is the result of a rapid supercooled liquid phase whose molecular activity is so low as to be unfavorable to nucleus formation and particle growth. Thus, the development of molecules to particles is relatively slight and therefore very little viscosity results. Conversely, the high viscosity resulting from a slow cooled mix initially held at 80°F. appears to be the effect of an optimum condition for the production of nuclei and their growth because this temperature favors molecular activity which permits equilibrium of kinetic energy.

If one may here introduce the hypothesis of Von Weimarn (14) that gelation of gelatin is an extreme case of crystallization, the above temperature (80°F) may be called the labile range in terms of crystallography. The range from 80° to 100°F. would be considered the meta-stable area because fewer crystals were formed as reflected by a lower viscosity in most instances. The figure 100°F., the melting (transition) point of gelatin according to Oakes and Davis (15), gives this point on the curves studied theoretical significance because it is linked with a distinct drop in viscosity in most instances. Obviously, a study in which the mix is held at the various temperatures and then rapidly cooled to the final temperature is necessary before accurate facts can be determined regarding the exact relation of crystallization of gelatin in ice cream to the rate of equilibrium.

Although the foregoing is of fundamental interest in explaining to a considerable degree the phenomenon of aging and variations in viscosity observed in the plant and laboratory, the seeker of the purely practical phase may note that considerable viscosity may be developed without seriously affecting the whipping property of the mix or the appearance of the melting frozen product. As to the question of bacterial growth, preliminary trials show that a significant rise in count does not begin until an initial aging temperature above 70°F. is employed. This is under condition of slow cooling and is apparently much more favorable to growth than a short holding period followed by a rapid cooling to 40°F.

CONCLUSIONS

1. The experimental work indicates that a greater part of the texture benefits resulting from aging an ice cream mix are associated with the viscosity imparted by gelatin.

2. A high initial temperature in the aging period favored the development of greater basic viscosity.

3. The maximum viscosity was found to be imparted when the mix was allowed to cool from 80–100°F. to 40°F. without agitation.

4. The whipping property was decreased as the viscosity increased.

5. The rate of melting was decreased as the viscosity increased. The manner of melt-down was also influenced by difference in viscosity.

6. This study indicates that the results of aging an ice cream mix may be dependent upon the factors affecting the crystallization of the gelatin portion of the product.

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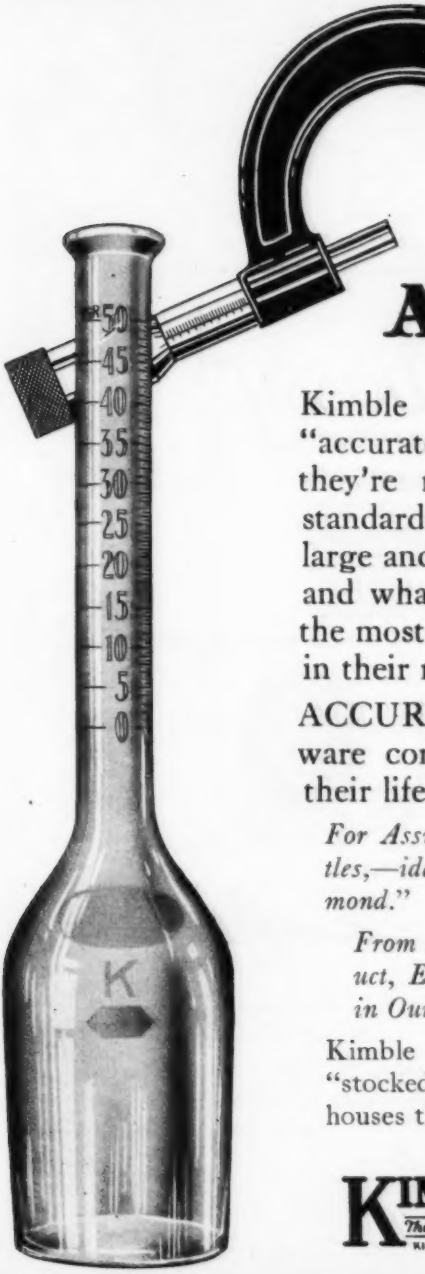

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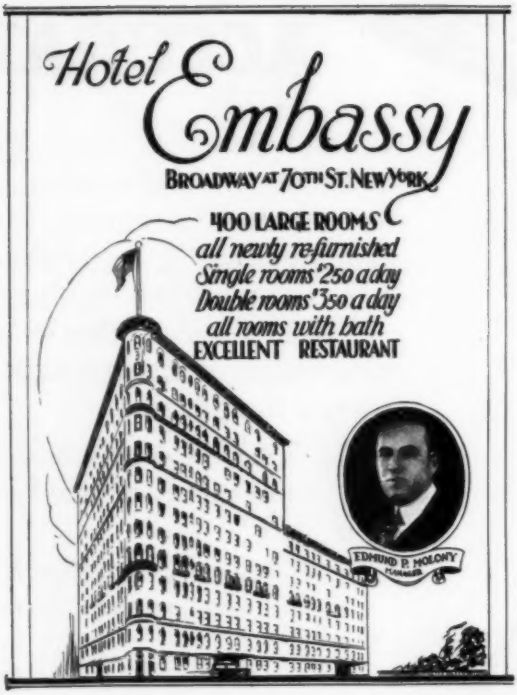

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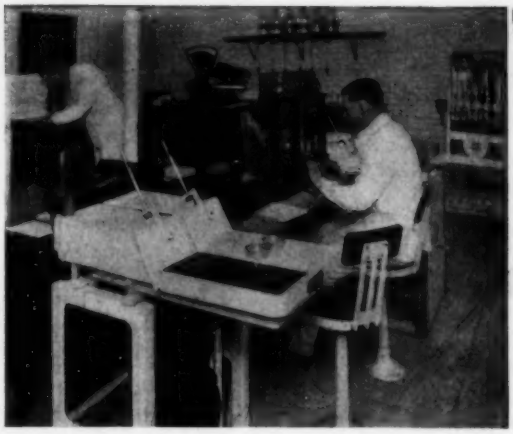

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